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INTERMEDIARY REACTIONS

INVOLVING (Na + K)-DEPENDENT ATPase

By

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ABSTRACT

The microsomal pellet obtained from the isolated mucosal cells of bladders of fresh water turtles contains a Na, K ATPase activity, detectable at 0°C as well as at 38°C.

Mg-dependent ^{32}p-labelling of the native and ouabain-treated protein by γ-^{32}p-ATP at 0°C amounts to 0.29 and 0.19 mmicromoles P/mgm protein/50 sec, respectively. In the native protein, addition of Na^+ increased this labelling by 158%; and addition of Na + K, decreased the labelling of the protein to the underlying Mg-dependent level. In the ouabain-treated protein, addition of Na increased Mg-dependent labelling by 220%; but addition of Na + K resulted in no change in labelling which remained at the Mg + Na dependent level. Mg-dependent ^{14}C-labelling of the native microsomal protein by ^{14}C-ATP at 0°C amounts to 0.72 mmicromoles/mgm protein/50 sec; and addition of Na^+ decreases the Mg-dependent labelling by 46%. The Na-induced decrement in microsomal binding of ^{14}C-ATP simultaneously with the Na-induced increment in binding of ^{32}p-ATP suggests the formation and breakdown of a sodium-sensitive E-ATP complex.

The catalyzed exchange reaction, ^{14}C-ADP + ATP $\rightleftharpoons$ ^{14}C-ATP + ADP, also found in the microsomal pellet, has an absolute requirement for Mg in the presence or absence of Na and/or of Na + K. The rate of exchange is reduced by Na and is increased by Ouabain over a wide range of Mg levels. The sodium-induced decrement of exchange is elicited in the presence and in the absence of Ouabain. In fact, sodium can entirely abolish the exchange reaction in the native microsomal proteins. The rate of exchange in the presence or absence of sodium is independent of

the pH over a wide pH range. Whereas oligomycin inhibits hydrolysis without affecting the exchange rate, NEM inhibits both processes. The nucleotide preference of the exchange rate with Mg alone was ATP > GTP > ITP > UTP > CTP; and with Mg + Na was ATP > CTP > ITP > UTP > GTP.

CHAPTER I

INTRODUCTION

The mucosal epithelium of the isolated bladder of fresh water turtles (Pseudemys scripta), under in vitro conditions, transports sodium, chloride, and bicarbonate ions against their respective electrochemical gradients ("uphill transport") from the urine-side (mucosa) to the blood-side (serosa) (1-5). Concomitantly, water is moved across the epithelium in the same direction, but down its gradient of chemical potential ("downhill transport"). The process of moving ions against the electrochemical potential gradient requires a source of metabolic energy and is generally referred to as "active transport."

Active transport of various ions, particularly sodium, occurs across single cell membranes in some instances (e.g. erythrocytes, muscle, nerve, and axone); and across an entire cell width including both cell membranes in other instances (e.g. frog skin, toad bladder, turtle bladder, renal tubule, salivary and sweat glands, gastro-intestinal epithelium, etc.). The active transport of sodium across a single membrane, called "transmembrane transport," is concerned with volume regulation, with transmission of excitation, but not with net movement of the salts or water in the steady state. On the other hand, active transport of sodium across a cell, or "transcellular transport" is concerned with absorption or secretion with the net movement of large amounts of salts, water and other materials in the steady state. Transcellular processes involve two membranes bounded by three fluid phases, while transmembrane processes involve one membrane bounded by two fluid phases.

The present study is concerned mainly with some biochemical aspects

of the process of active transport of the sodium ion across the turtle bladder--a process which has been well described in terms of electrophysiological and transport parameters (1-5), but not in terms of biochemical parameters. Whereas biochemical studies involve procedures which destroy the cell, a large body of recent work in ATPase activity has provided additional insight into the nature of sodium transport in several systems. In particular, the Na + K-stimulated moiety of ATPase has been found in a wide variety of sodium-transporting tissues (6-10). Accordingly, the present study was focused on the cation-sensitive ATPase activity in the microsomal (or membrane) fraction of the mucosal epithelial cells of the turtle bladder.

Biochemical studies of membrane fragments are designed to determine the ion-specificity of the membrane bound protein, the carrier function which mediates translocation of the transportable ion, and the mechanism for delivery of free energy into the ion-carrier complex. Although the molecular nature of ion-specificity and of the carrier operation in membranes remains unknown, much useful information of an indirect nature has been obtained in recent years.

In many cells, active sodium transport apparently depends specifically on ATP as a source of energy (11-15). Skou (16) first suggested a possible link between (Na + K)dependent ATPase and transport in crab nerve. Subsequently, Post (17) and others (18) found that the kinetic pattern of (Na + K) dependent ATPase activity of microsomes versus sodium was similar to that of Na transport versus internal sodium concentration in red cell ghosts. The hydrolysis of ATP and Na transport in red blood cells both require K in external medium and Na within the cell. Moreover,

Hoffman (11) has shown that ATP (and not other nucleotides) on the inner surface would initiate Na transport out of erythrocyte ghosts incubated without other substrates. Cardioactive steroids (e.g. ouabain) are effective inhibitors in the external medium (17, 19) in erythrocytes; or analogously, in the fluid phase toward which Na is transported in other tissues (6, 14, 20).

The (Na + K) dependent ATPase is closely associated with cell membrane fragments. These fragments, defined operationally as the sediment obtained after centrifugation at 65,000 g, are called "microsomal" fractions (14,16). Several attempts, using histochemical means, have been made to locate Na + K ATPase in portions of sarcoplasmic reticulum, but such data have been conflicting (21,22) due to the inhibitory effects of heavy metals (23) and tissue fixatives (24, 25).

The microsomal fraction isolated from the mucosal cells of the turtle bladder, a known sodium transporting system (1, 2), possesses a (Na + K) stimulated, ouabain inhibited ATPase activity (1, 2), satisfying five out of eight criteria of Skou (14) for identification and isolation of a transport system. The five criteria are: a) the enzyme has been found in a system which transport sodium; b) the enzyme activity detected in bladder cells does catalyze the hydrolysis of ATP, which is theoretically capable of transferring the free energy of hydrolysis into that needed for cationic transport; c) there is a parallel inhibitory effect of cardiac glycosides on the activity of the isolated enzyme extract and on the sodium transport in the intact cell; d) the pattern of increasing ATPase activity versus the concentration of Na + K in the microsomes resembles qualitatively the pattern of increasing sodium transport versus the concentration of mucosal sodium in the intact bladder; and e) the Na + K ATPase is located in the

microsomal fraction of mucosal cells (14).

Attempts at further physical or chemical fractionation of the microsomal proteins have resulted in inactivation rather than purification of the enzyme. The inactivation during purification has been ascribed in part to the removal of phospholipids which are apparently required for the structural integrity of the enzyme. The molecular weight of the enzyme protein, estimated from data on sedimentation after solubilization with different detergents, and from data on radiation inactivation, varies from 670,000 to 250,000 (26-29). For the (Na + K) dependent ATPase, magnesium and sodium ions are absolutely required. In fact, Mg is needed not only for activation of the enzyme, but as part of the substrate, which has been shown to be Mg-ATP (30). The requirement for potassium ions can be satisfied partially with ions of lesser stimulative activity (e.g., $NH_4^+ > Rb^+ > C_s^+ > L_i^+$). Maximal activity generally occurs at physiological levels of pH (7.3) and ionic strength (3 mM ATP, 3 mM Mg, 50-100 mM Na and 10-20 mM K). Divalent cations other than Mg, Ca and Cu, have inhibitory effects on ATPase activity (6, 14).

It has been suggested that the enzyme-stimulating ions (Na, K, Mg) act as effectors to modify the enzyme activity (31, 32). Homotropic or heterotropic effects may be the kind of modification produced by the aforementioned ions. Two or more sites on the enzyme for each of the effectors, Na and K, have been postulated (6, 31-33). This multi-site postulate is consistent with data on the "sodium transport" efficiency of about 2-3 Na transported per mole of ATP hydrolyzed in several tissues (34).

The ATPase-catalyzed hydrolysis of ATP ($ATP + H_2O \longrightarrow ADP + P_i$), like any catalyzed reaction, involves the formation of one or more intermediary

complexes with the enzyme before the formation of the final reaction products. One of the intermediary reactions, in many cell types, is the formation of a phosphorylated intermediate which has properties of a high energy phosphate complex, and which is usually detected as the ^{32}P-labelled acid-stable protein after incubation of the microsomes at 0°C with γ-^{32}P-labelled ATP (35). The sodium sensitivity of the phosphorylation (6, 14, 36, 37), and the potassium and ouabain-sensitivity of the dephosphorylation (6, 14, 38, 39) have been considered relevant to the sodium transport mechanism. Of further relevance, was the finding of sodium-sensitivity in the exchange reaction between ATP and ADP--an enzymatically catalyzed exchange occurring concomitantly with the same microsomal proteins as those catalyzing overall hydrolysis, phosphorylation and dephosphorylation (6, 14, 40).

The available evidence on the first step in the overall hydrolysis implies that the ADP moiety of the ATP molecule is cleaved from the γ-phosphate simultaneously with the bonding of the γ-phosphate to the protein. However, the first intermediary complex formed should be that between the enzyme and ATP, but this has not yet been demonstrated directly.

The purpose of the present work was to characterize certain intermediate steps in the ATPase-catalyzed hydrolysis of ATP, with the ultimate aim of correlating such steps with the physiological properties of the sodium transport mechanism in the intact turtle bladder. Details studied included: the rate and cation-dependency of ^{32}P-labelling and of ^{14}C-labelling of microsomal proteins which had been incubated with γ-^{32}P-ATP and with ^{14}C-ATP respectively; chromatographic measurement

of the rate and cation dependency of the conversion of ^{14}C-ADP to ^{14}C-ATP (exchange rate) in the presence of the microsomal proteins via the reaction,

$$^{14}\text{C-ADP} + \text{ATP} \overset{\text{Mg}}{\rightleftharpoons} {}^{14}\text{C-ATP} + \text{ADP},$$

wherein the γ-phosphate of ATP is transferred to ^{14}C-ADP in a transphosphorylation step. The effects of certain inhibitors (i.e., ouabain and NEM); and the nucleotide specificity of the protein binding process and of the exchange rate were studied.

An integrated model is presented in an attempt to understand the entire ATPase system involving intermediate complex formation, exchange reaction and hydrolysis.

The mounting evidence herein and elsewhere for a variety of tissues points to the claim that one of the chemical mediators of sodium transport (e.g. the translocator or the metabolic energy converter) may be an enzyme which can function as an ATPase.

CHAPTER II

Methods

A. Materials

ATP, GTP, UTP, ITP, CTP and ADP as Tris-salts; disodium ATP, ouabain, NEM (N-Ethyl maleimide), oligomycin, pyruvate kinase, lactic dehydrogenase, phosphoenolpyrüvate, L-histidine HCl, and imidazole grade I were obtained from Sigma Chemical Co., St. Louis, Mo. Gamma-labelled ^{32}p-ATP, 8-^{14}C-ADP as the ammonium salts, 8-^{14}C-ATP, as the sodium salt, and $^{32}p_i$ in 0.02 N HCl were obtained from ICN (International Chemical and Nuclear Corporation, California).

B. Isolations of Microsomal Fraction

The procedure used for separating and isolating mucosal cells was that recommended by Solinger et al. The necks of the ten bladders, in the form of closed sacs, were tied to the outlets of Luer lock syringes and immersed in Ca-free Ringer solution containing bicarbonate, 17 mM and EDTA, 2mM. In some cases, choline Ringer, and in others, sodium Ringer solution was used. The complete chemical composition of both Ringer solutions has been described (1). The mucosal surface of each bladder was washed 3-4 times through the Luer lock syringe with Ringer solution obtained from the same stock as that in which the bladder was immersed. The sacs were then filled with 15-25 ml. of Ca-free Ringer with EDTA immersed. in 1000 ml. of Ringer solution, and incubated for 30-40 minutes at room temperature (usually 25°C). During incubation, the external (serosal) bath was gassed with 99% O_2. Then the walls of the sacs were rubbed gently against one another for 2-3 minutes, a maneuver which released the epithelial cells into the mucosal fluid. The cell-containing

mucosal fluid was removed from the sacs and subjected to the centrifugation procedure described by Fig. 1.

Fig. 1 is a modified flow sheet diagram of the procedure for obtaining the various centrifugal fractions from isolated mucosal cells. Low temperatures (0-2°) were maintained throughout the entire procedure. Isolated mucosal cells, obtained by the EDTA treatment of 10 bladders and suspended in a total volume of about 250 ml of Ringer solution, were cooled and centrifuged at 10,000 g for 15 minutes in the Spinco-refrigerated ultracentrifuge. The pellet of packed cells was then suspended in 10-15 ml of 0.25 M sucrose, 1 mM EDTA, and 0.1% DOC, homogenized in a Dounce glass homogenizer, (at this point an aliquot of the crude cell homogenate was removed, diluted and assayed for ATPase activity if desired), and the suspension was diluted to 90-100 ml using the sucrose-EDTA-DOC solution. This cell homogenate was centrifuged at 10,000 g for 15 min. The resulting pellet was removed, re-homogenized, resuspended in sucrose-EDTA-DOC solution, and centrifuged at 10,000 g for 15 minutes. The supernatants obtained from both of the 10,000 g centrifugations were combined and centrifuged at 65,000 g for 60 minutes. The pellet so obtained was suspended in 10-15 ml of 0.25 M sucrose, 1 mM EDTA, and 0.05% DOC, homogenized, and diluted in 90-100 ml of the sucrose-EDTA-0.05% DOC solution. The suspension was then centrifuged at 65,000 g for 20 minutes. The resulting pellet was resuspended in sucrose, EDTA, and 0.05% DOC, homogenized, and centrifuged again at 20,000 g for 20 minutes. The supernatants obtained from both of the 20,000 g centrifugations were combined, and centrifuged at 65,000 g for 60 minutes. The pellet obtained in the last centrifugation (65,000 g for 60 minutes) was suspended in 15-20 ml of 1 mM EDTA, stored at -10° and used as an

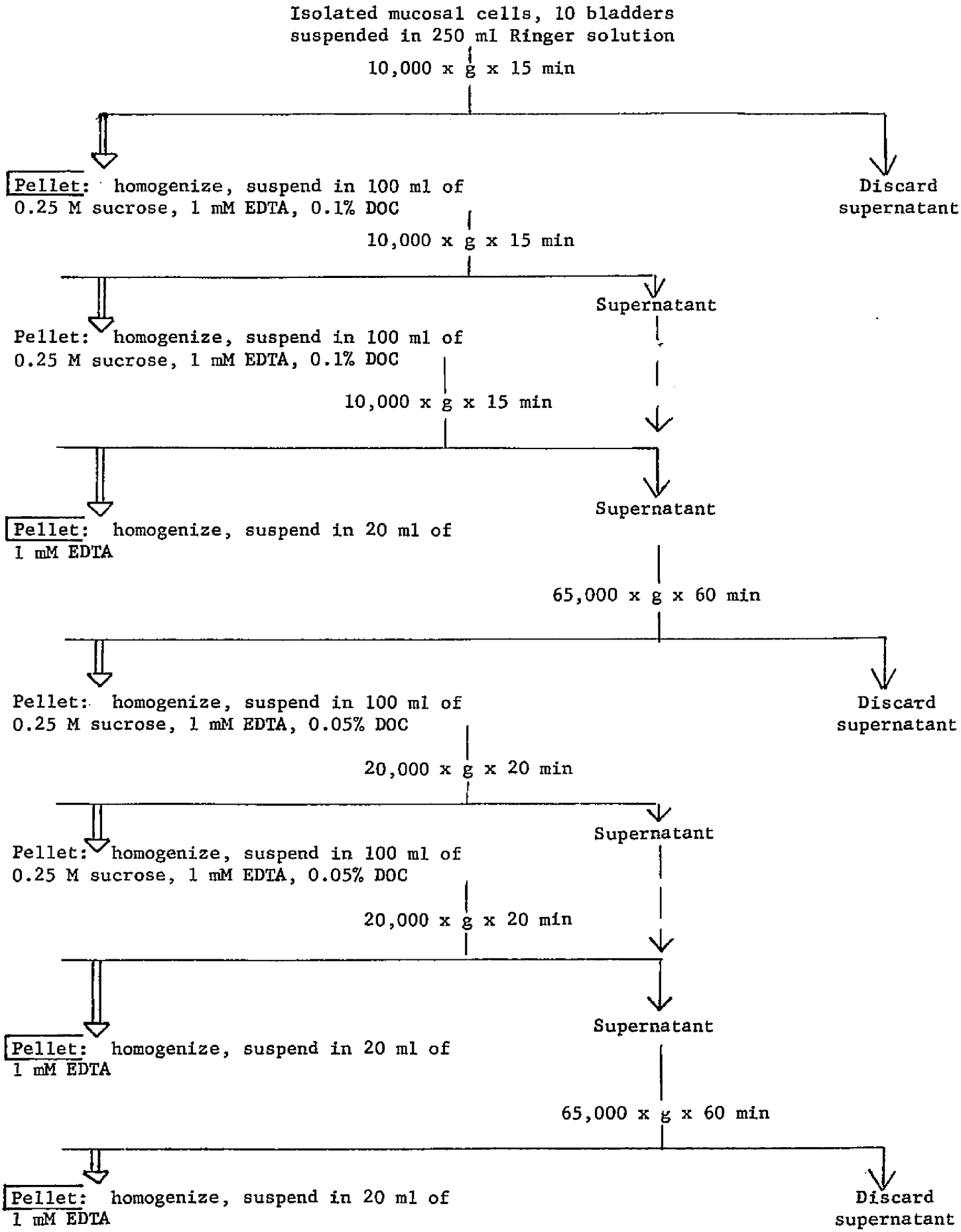

Fig. 1. Flow-sheet diagram of procedure used for obtaining centrifugal fractions from isolated mucosal cells.

enzyme source for all the experiments reported here. In some cases, the pellets obtained at 10,000 g and 20,000 g, as well as all of the supernatants were examined for ATPase activity.

C. Assay of ATP hydrolysis

The method used for rapid spot checks of ATPase activity in different fractions was the micro-technique of Lowry et al (42) as recommended by Albers et al (43). In this technique, the ADP being formed during hydrolysis was allowed to react with an excess of PEP, NADH, pyruvate kinase, and lactic dehydrogenase so that the rate of oxidation of NADH (monitored continuously in the cuvette of a Zeiss PMQ II spectrophotometer) was directly proportional to the rate of formation of ADP. This method is not generally recommended for routine use in potassium-sensitive reactions (43) because the pyruvate kinase activity itself is K sensitive (44,45).

The method used routinely was as follows: Microsomes were diluted as required. In the standard assay procedure, final concentrations in the incubation mixture were as follows: 3 mM γ-^{32}P-ATP (specific activity 1 x 10^5 cpm per µmole), 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH = 7.3) as a buffer, 0.1 mM EDTA-tris, inhibitors when indicated, and 4 µg of enzyme protein in a final volume of 100 µl.

The assay media less ATP were allowed to pre-incubate in tubes (5 x 50 mm) ca one-half hour at 30°C before initiating the hydrolysis by addition of the γ-^{32}P-ATP. The resulting mixture was incubated at 30°C (or in some cases at 0°C) for 10 to 20 minutes prior to termination of the reaction by addition of 25 µl of 25% (w/v) of perchloric

acid (PCA).

After centrifugation of the PCA-treated mixture at 15,000 g for 15 minutes at 0°C, the supernatant fluid was analyzed for either inorganic ^{32}P or, in some cases, inorganic phosphate, by a modified Berenblum and Chain technique (46). An aliquot (100 µl) of the supernatant was removed and mixed with 30 µl of 5% (w/v) ammonium molybdate in 4N H_2SO_4. Next, 100 µl of isobutanol was added for extraction of the phosphomolybdate complex from the aqueous to the alcoholic phase--a procedure accomplished by vibratory buzzing of the tubes for no less than 25 sec. Such a period of vibration provided an optimal extraction yield under these conditions. At this point, the procedure was considered as terminated with respect to radioisotopic counting of $^{32}P_i$, since no color development is required for such an end-point measurement. In other cases, the procedure was carried through the steps leading to color development for measurement of non-radioactive inorganic phosphate.

After the vibration maneuver, the alcoholic and aqueous phases were separated by centrifugation at 1000 g for 5 minutes at 0°C, after which an aliquot (50 µl) of the isobutanol layer was added to the naphthalene-dioxane scintillation counting fluid (43) for measurement of ^{32}P in a Beckman scintillation counter. The amount of ATP hydrolyzed was calculated from the data on ^{32}P counts and specific activity of $AT^{32}P$ in the reaction mixture. The rate was normalized with respect to the amount of microsomal protein, determined by the Lowry method (47).

(Na + K) stimulated ATPase is defined as that activity measured in the presence of Mg + Na + K, less than that measured in the presence of Mg alone. The Na + K stimulated part is inhibited by ouabain, the Mg-dependent part is not. Therefore Na + K stimulatable ATPase is often called ouabain-sensitive ATPase, and Mg-dependent ATPase is often called ouabain-insensitive ATPase.

D. Intermediate Measurements

1. Assay of ^{32}P-labelling of microsomal proteins

Microsomal protein, 0.04 to 0.20 mg, was incubated in an ice bath for 50 sec. (unless otherwise indicated) in a medium containing 1 mM $AT^{32}P$ (specific activity, 10^5 cpm per mμmole), 85 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH = 7.3) as buffer, 0.1 mM Tris-EDTA, KCl and inhibitors when indicated, in a final volume of 100 μl. Reactions were terminated by addition of 100 μl of 10% (w/v) PCA, after which the mixture was centrifuged at 15,000 g for 15 minutes at 0°C. (At this step, hydrolysis at 0°C was measured when desired by removing an aliquot of the supernatant and treating it as described above).

The PCA precipitate was washed twice with 200 μl aliquots of a cold (0°C) solution containing 5% PCA, 0.05 M Na_2ATP, and 0.05 M KH_2PO_4. The entire 400 μl of the resulting suspension was filtered by suction through millipore paper with a pore size of 0.45 μ; after which an additional 25 ml of the same cold washing solution was placed on the same paper and filtered by suction.

The volume desired for washing was pre-determined by increasing the volume of washing solution (from 5 ml to 50 ml) in order to determine at what point further increases in volume produced no change in

radioactivity of the filtrate. Filter paper, so treated, was immersed into 10 ml of toluene scintillation counting solution, and radioactivity was measured in the Beckman scintillation counter.

Two types of blanks were obtained: the first, by filtering and washing the microsome-free incubation mixture (Filter Control); and the second, by filtering and washing the same incubation mixture containing microsomes previously denatured by treatment with PCA, (PCA-Killed Control). In all cases, the radioactivity of the Filter Control was much less than that of the PCA-Killed Control.

Another type of blank was only obtained (in two experiments, but not routinely), by filtering and washing the same incubation mixture containing microsomes previously denatured by immersing the mixture into a boiling water bath for 3-5 minutes, (Heat-Killed Control). It was found that the average value of labelling of the heat-killed microsomes was the same as that of PCA-killed microsomes. Consequently, the latter was used routinely as the base line Control for nonspecific labelling in all of the experiments to be reported herein.

The use of millipore filter for the washing of microsomal protein was chosen in preference to the consecutive washing and centrifugation steps used by others (37). This was because the measured differences between ^{32}P-labelling of the microsomes and that of the same microsomes, pre-treated with PCA (i.e. PCA-Killed Control) were greater in the millipore method than in the consecutive centrifugation method. The amount of protein labelling was estimated from the counts of ^{32}P in the protein together with the specific activity of γ-^{32}P-ATP.

2. Assay of ^{14}C-ATP-labelling of protein

Microsomal pellets, 0.05 to 0.20 mg, were incubated in the same medium as described above, except that ^{14}C-ATP, 1 mM (specific activity 10^5 cpm per mμmole), instead of ^{32}P-ATP was used.

3. $^{32}P_i$-labelling of protein

Inorganic phosphate ($^{32}P_i$), 5 mM, (specific activity 10^5 cpm per mμmole), instead of ATP, 1 mM was added to the standard microsomal fraction and carried through the entire procedure described for ^{14}C and ^{32}P labelled ATP in order to determine the rate of $^{32}P_i$ labelling of the microsomal proteins.

E. Exchange rate measurements

1. Transphosphorylation Assay (Exchange rate)

The standard incubation mixture consisted of the following final concentrations: 5 mM Tris-ATP, 0 to 3 mM $MgCl_2$, 0 to 125 mM NaCl 1.6 mM ^{14}C-ADP (specific activity 1 x 10^6 cpm per μmole), 40 mM Tris-HCl (pH 7.3) as buffer, 0.1 mM EDTA-tris and about 1 to 5 μg of microsomal protein in a total volume of 30 μl. The exchange assay can be quantitated only when ATP and ADP remain reasonably constant (48). This was achieved by omitting K, and checked experimentally by determining the percentage of the Mg-dependent hydrolysis. Results showed that 12% or less of ATP was hydrolyzed under the conditions used in the routine assay.

The samples, in tubes (5 x 50 mm), were incubated at 38° for 10 minutes. The reaction was stopped by adding 5 μl 10% (w/v) formic acid in methanol. Preliminary tests showed that this treatment gave less Mg-dependent hydrolysis than did the treatment of placing the rack of tubes in a boiling water bath for 2 minutes prior to placing it in

an ice bath (40). Perchloric acid was not used to terminate the reaction because of its interference with the thin layer chromotography. Each sample, along with similar volumes of standard known nucleotides, 3 to 5 µl, was applied to the chromatographic plates. After separation on the plates, counting was performed according to the procedure used by Fahn et al (40). Under the aforementioned assay conditions for measuring the exchange rate, no ^{14}C-AMP formation was detected, thus eliminating contribution of adenylate kinase activity to the exchange rate. Exchange rate was calculated from the specific activity of the ^{14}C-ADP; and expressed in terms of the ^{14}C-ATP formed as a percentage of the total ^{14}C-ADP present initially.

2. Assay of the exchange rates of ITP, CTP, GTP, and UTP, with ^{14}C-ADP

The composition of the incubation mixture, in millimolar concentrations, was as follows: 5 mM Tris-ITP; or (of the tris salts of CTP, GTP, or UTP) when indicated; 3 mM Mg Cl_2; 0 or 85 mM NaCl as indicated; 1.6 mM ^{14}C-ADP; 40 mM Tris-HCl (pH 7.3) as the buffer; 0.1 mM EDTA-tris; and approximately 8 µg of microsomal protein in a total volume of 30 µl. Aliquots of the reaction mixture were run on thin layer chromotography and assayed for the ^{14}C-ATP formed. The rest of the assay procedure was as described under the section on transphosphorylation assay.

CHAPTER III

THEORETICAL CONSIDERATIONS

On the basis of the data to be presented here concerning microsomal binding, hydrolysis, and exchange reaction, the three processes have been included into a single reaction model (Fig.2). The reaction model is displayed preceding the results section to aid the reader and to serve as a guideline for the data to be introduced.

Many models can be rendered compatible with data on hydrolysis alone; with data on microsomal binding, or with data on the exchange reaction alone, or with data from any two of the three types of processes. However, only a limited number of models can be rendered compatible with data obtained independently from all three different processes -- hydrolysis, binding and exchange. The ultimate ideal objective in this, as in any scientific study, is to approximate the situation wherein one theoretical model can account for a maximal variety of independently obtained, qualitatively different data on the same system. Complete discussion of the reaction model will be introduced when the exchange reaction data are presented.

Figure 2 is a reaction model for ATPase activity, where E and E' denote different forms of the enzyme. The Mg-dependent hydrolysis is assumed to proceed through reactions 1 to 3 inclusive; the Na + K-dependent hydrolysis through reactions, 1, 4, 5 and 6; and E-ATP is assumed to be the common precursor for both paths. The original form of the enzyme is called E, and the sodium modified enzyme is called E'.

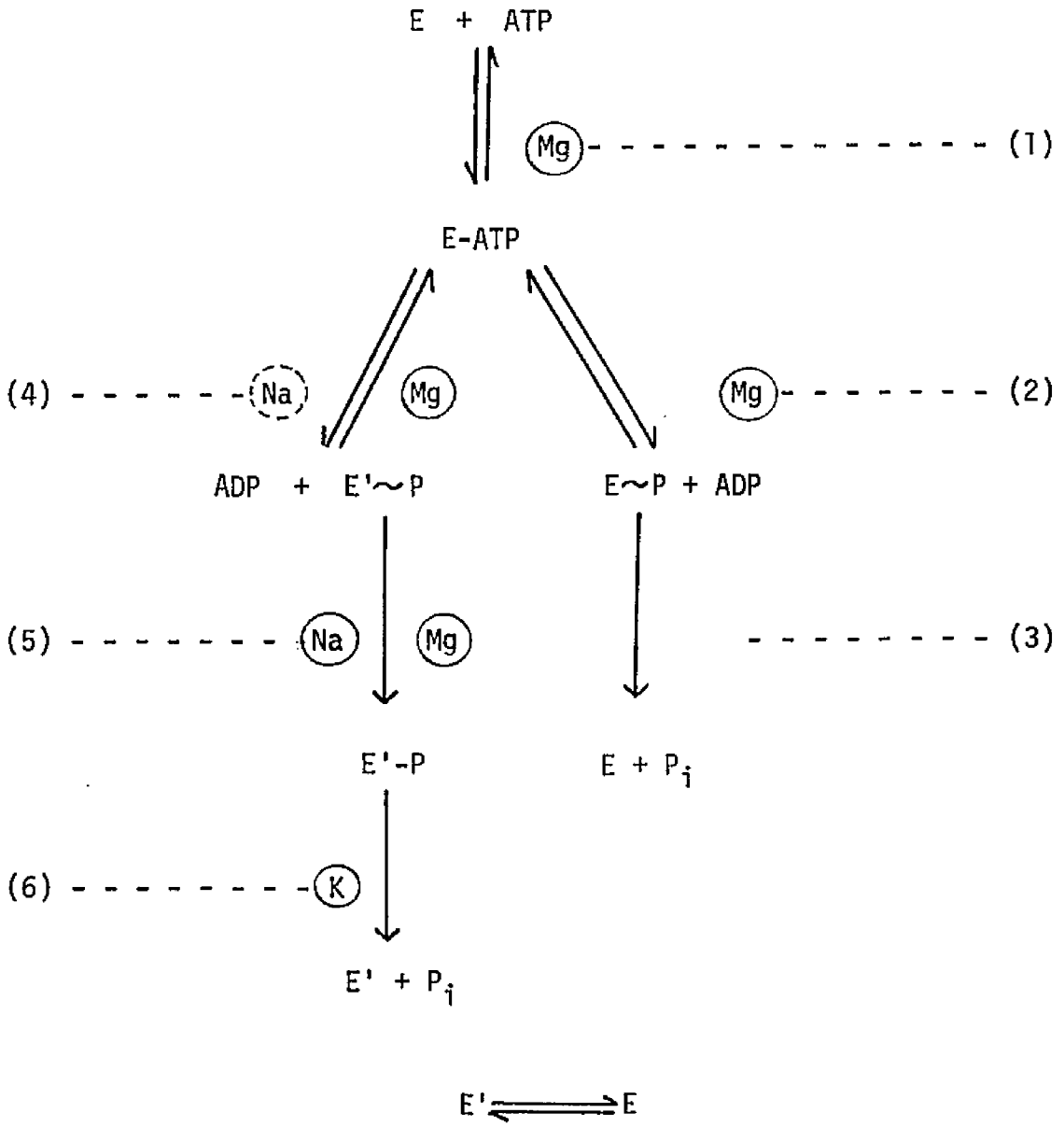

Fig. 2. Reaction model for ATPase activity.

Data to be presented from turtle bladder microsomes, suggest that the main site of action for sodium is at reaction 5; and the minor site, at reaction 4.

Parenthetically, in microsomes obtained from other tissues (6, 10), sodium apparently acts mainly at reaction 4.

CHAPTER IV

RESULTS

A. ATPase activity with γ-^{32}P-ATP

Table 1 presents values derived from $^{32}P_i$ measurements, on the hydrolytic activity of the native and of the ouabain-treated microsomal pellet in one out of 10 similar experiments under the "cationic" conditions designated in the first column. As expected, the simultaneous addition of Na^+ and K^+ to the incubation mixture (3rd row) increased the Mg^{++}-dependent activity (first row) by ca. 100% in the native microsomes -- an effect which was completely blocked in the microsomes which had been pre-incubated with ouabain (4th and 5th rows).

Such data, derived from the use of γ-labelled ^{32}P-ATP were consistent with our previous data, derived from the use of non-radioactive ATP (Y.E. Shamoo and W.A. Brodsky, unpublished data); and consistent with more recent data of others (49) who used an ADP assay for measuring ATPase activity in the microsomal pellet from turtle bladder.

B. Microsomal Binding

1. ^{32}P-labelling from γ-^{32}P-ATP

Table 2, presents values, from one representative experiment (see table 3 for massed data), on the 50 second rate of formation of ^{32}P-phosphoprotein in the native and in the ouabain-treated microsomes under the ionic conditions designated in the first column and under the buffer conditions at pH 7.3 designated in the second and third columns.

In this, as well as in all other experiments, the amount of protein-labelling after incubation of PCA-killed microsomes was substracted from

Conditions for Hydrolysis	Specific Activity (μmoles/mg/hr)	Relative Specific Activity
Mg	11.0	1.00
Mg + Na	10.6	0.96
Mg + Na + K	21.7	1.97
Mg + Ouabain	10.6	0.96
Mg + Na + K + Ouabain	10.4	0.95

Table 1. Na, K ATPase activity. Each tube contained ca. 4 mgm of microsomal protein (in the native state or pre-treated with ouabain as described in Methods) together with the appropriate cations listed in the first column. Composition of the incubation mixture, in terms of millimolar concentration, was: Na, 85; K, 15; Mg, 3; Tris-γ-^{32}P-ATP, 3 (specific activity = 10^5 cpm/μmole; Tris-EDTA, 0.1; and ouabain, 1.0. Final pH, 7.3 and final volume, 100 μℓ. After incubation at 37°C for 15 minutes, the reaction was stopped by adding 25μliter of cold 25% (w/v) perchloric acid, and the inorganic phosphate ($^{32}P_i$) was extracted by the method of Berenblum and Chain (17).

In another set of experiments in this laboratory on Na, K, ATPase from the same tissue, the mean values of a large mass of data were similar to those shown here. (Shamoo, Y.E. and W.A. Brodsky, Biochim. Biophys. Acta, In Press, 1969)

	Conditions for Labelling	mμmoles P incorporated/ mg Protein/50 Sec.	
		Tris-HCl as Buffer	Imidazole-Histidine as Buffer
Native Enzyme	Mg	0.57	0.99
	Mg + Na	1.43	1.22
	Mg + Na + K	0.36	0.50
Ouabain Treated	Mg	0.27	0.38
	Mg + Na	1.36	0.27
	Mg + Na + K	1.22	0.67

Table 2. Amount of binding in the native and ouabain-treated microsomes under different ionic conditions. Each tube contained approximately 200 μgm of microsomal protein in a native or ouabain-treated system containing either 50 mM Tris-HCl or 50 mM imidazole-histidine (pH = 7.3) together with the appropriate cations listed in the first column. Final concentration of each constituent of the incubation mixture was: 85 mM Na, 15 mM K, 3 mM Mg, 3 mM γ-^{32}P-ATP (specific activity 10^9 cpm/μmole, 0.1 mM Tris-EDTA, and 1 mM ouabain. Final volume, 100 μl; temperature 0°C, time of incubation, 50 sec. Reaction was terminated by adding 100 μℓ 10% (w/v) cold perchloric acid (final concentration 5%).

that obtained after incubation of enzymatically active microsomes. The magnitude of the non-specific labelling by PCA-killed microsomes varied from 10 to 80 per cent of that attributable to the Mg-dependent labelling found after incubation for 50 seconds at 0°C.

In terms of raw cpm, the order of magnitude of determinations were as follows: background, 40; "filter control", 100; PCA-killed control, 240; enzymatically active microsomal samples after 50 seconds of incubating at 0°C in the presence of Mg alone, 500-1000, and in Mg + Na, 750-1500. The magnitude of the enzymatically active relative to that of the non-specific labelling was at least as large as that usually reported by others in this field.

a. Effect of cations and ouabain on the phosphoprotein (Table 2)

In the native microsomal fraction, the addition of Na increased the Mg-dependent phosphorylation in both buffer systems; while the simultaneous addition of Na + K decreased the Mg-dependent phosphorylation of the protein in both buffer systems.

In the ouabain-treated microsomal fraction buffered by Tris-HCl the addition of Na induced an increase in the Mg-dependent rate of ^{32}P labelling of the protein while the simultaneous addition of Na and K also increased the Mg-dependent labelling to the same extent as did Na alone.

In the ouabain-treated microsomal fraction buffered by imidazole-histidine, the results differed from those obtained with Tris-HCl buffering. For example, addition of Na did not stimulate the Mg-dependent rate of labelling of protein, and the simultaneous addition of Na and K increased the Mg-dependent labelling to a greater extent than did Na alone.

In the native microsomes, the effects of Na and K on ^{32}P-labelling of protein were similar to those reported by Fahn et al using Tris buffer (50) and by Post et al using imidazole-glycylglycine buffer (37).

In the ouabain-treated microsomes, the effects in one buffer were different from those in the other; and somewhat different from but not necessarily inconsistent with those reported by others (14,37,50).

For example, in Tris buffer, the sodium-dependent labelling was not appreciably affected by ouabain as seen in the imidazole-histidine buffered system herein, and as seen elsewhere in Tris and in imidazole-glycylglycine buffered systems (37) subjected to ouabain treatment.

In our hands, the presence of ouabain in either buffer system apparently blocked the K-dependent dephosphorylation of the protein as has been reported in other tissues (6,14,43).

Since the aforementioned differences were based on but one experiment, we decided to perform a series of experiments on native and ouabain-treated microsomes in order to establish the average behavior of several pools of microsomes.

Table 3 presents mean values and statistical parameters for the cation-sensitive rate of ^{32}P-labelling of native and of ouabain-treated microsomal proteins. Values are normalized with respect to the Mg-dependent labelling rate, 0.29 mμmoles/mg protein/50 sec. taken as 100%.

In the native microsomal protein, addition of Na increased the mean labelling activity by 158% in 7 experiments ($P < 0.02$); and addition of Na + K produced little if any significant change ($P > 0.6$) above the Mg level of labelling. Expressed in other words, the addition of K to the Mg + Na loaded protein induces a significant ($P < 0.02$) stripping of the protein-bound ^{32}P.

Conditions for Labelling		Percent Phosphorylation (Mg^{++}-dependent as 100%) Native	Ouabain
A.	Mg	100 ± 21 n = 7	64 ± 16 n = 6
B.	Mg + Na	258 ± 49 n = 7 B - A P < 0.02	205 ± 47 n = 4 B - A P < 0.05
C.	Mg + Na + K	126 ± 49 n = 4 C - A P > 0.6	203 ± 41 n = 4 C - A P < 0.05

Table 3. ^{32}P-labelling of native and ouabain-treated microsomes incubated with γ-^{32}P-ATP for 50 seconds at 0°C under the cationic conditions designated in the first column. The probability values (P) are derived from the mean values and standard errors of the individual paired differences, $(Mg + Na)_i - (Mg)_i$ or B − A; and $(Mg + Na + K)_i - (Mg)_i$ or C − A. Composition and volume of incubation mixtures are the same as has been described for Table 2.

In the ouabain-treated microsomal protein, the ^{32}P labelling rate with Mg alone was 64% of the corresponding rate in the native protein.

A surprising finding in the ouabain-treated mixture was the fact that ouabain failed to change the sodium-dependent increment of the Mg-dependent ^{32}P labelling of protein.

The labelling rate found with Mg + Na + ouabain (205% of the native Mg-dependent level) was 220% greater than that found with Mg + ouabain (64% of the native Mg-dependent level). This Na-dependent increment in the ouabain-treated microsomes of turtle bladder was greater than that reported by others in similarly treated microsomes from different tissues (14,37,49).

As expected, the K-induced stripping of ^{32}P from the Mg + Na treated microsomes was completely blocked by ouabain ($P > 0.6$). Such a blocking of dephosphorylation has been found in other microsomal systems treated with ouabain (6,14,37,50).

b. NEM effect on phosphoprotein

In addition to the experiments of the effects of ouabain on ^{32}P labelling, four experiments were performed to determine the effects of NEM on ^{32}P-labelling in the presence of Mg and Na. Mean values ± S.E. of ^{32}P-labelling of the NEM-treated microsomes as percentages of ^{32}P-labelling of the native protein with Mg alone were as follows:

(1) 134 ± 73 in the presence of Mg + NEM, which was statistically the same as 100% in the presence of Mg alone; and

(2) 396 ± 124, in the presence of Mg + Na + NEM which was statistically greater than that in the presence of Mg alone -- indicating that the sodium-stimulation of ^{32}P-labelling of microsomes remained intact in the presence of NEM.

2. Substrate Specificity

ITP is almost as good a substrate as is ATP for the following: Na + K-dependent hydrolysis of nucleotides in microsomes of crab nerve (14, 36) and of turtle bladder (Y. E. Shamoo, and W. A. Brodsky - unpublished), and Na-dependent ^{32}P-labelling of microsomal proteins from crab nerve (14, 36); for the Mg-dependent exchange between nucleoside diphosphates and nucleoside triphosphates in microsomes from crab nerve (14, 36) and rat brain (51).

On the other hand, ATP is apparently the preferred substrate for the Na + K-dependent ATPase, as well as for Na-dependent phosphorylations and exchange reactions in the electric organ of the eel (40, 50, 52).

The nucleotide preference for the Na-dependent ^{32}P-labelling of microsomal proteins of bladder epithelial cells was determined for ATP, ITP, GTP, and UTP. The technique was to determine first the rate of ^{32}P binding from 1 mM ^{32}P-ATP, and then determine the effect of such binding by adding 5 mM concentrations of other nucleotides to the incubation mixture.

Figure 3 presents, in columnar form, mean values and standard errors of the binding activity of the microsomal proteins for five nucleotides in 4 separate experiments (each including the 5 nucleotides) on a single pool of microsomes. Results showed the following order or nucleotide preference: ATP > UTP > GTP > ITP > CTP. Thus, ^{32}P-ATP binding corrected for the presence of equimolar amounts of ITP, CTP, GTP and UTP was 12, 16, 1 and 1%, respectively. This can only be considered as a crude estimate of the nucleotide preference, and to this extent, ATP is largely the preferred substrate in the phosphorylation reaction--a finding in harmony with phosphorylation data in the electric organ of the eel (40,50), but not with similar data on phosphorylation from crab nerve (14).

3. Hydrolysis at 0°C

Data on microsomal ATPase activity has been derived from measurements at 38°C; and the addition of Na + K to the Mg-containing system

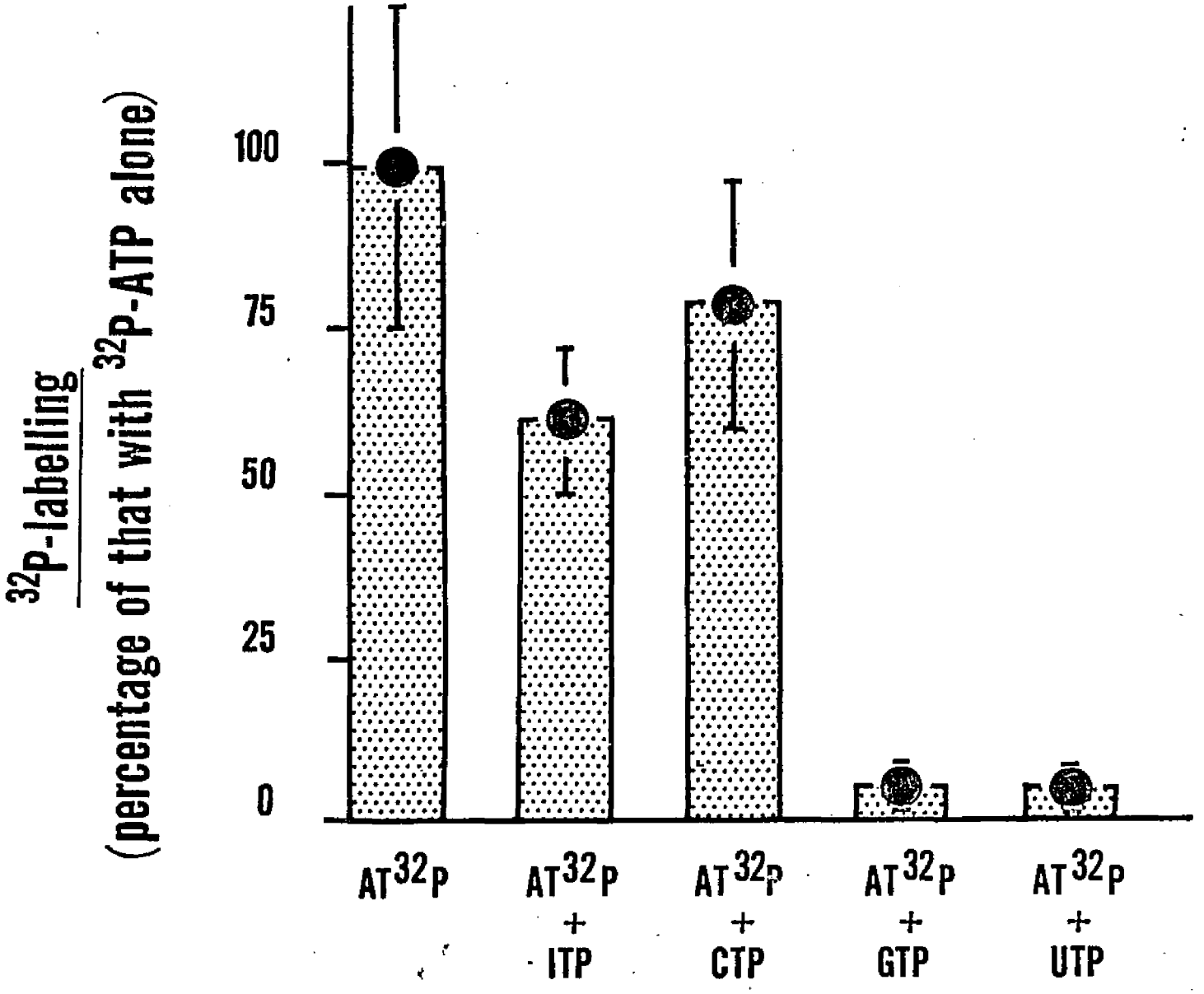

Fig. 3. Effect of ITP, CTP, GTP, and UTP (5 mM ea.) on relative ^{32}P-labelling in 4 experiments on a single pool of microsomal proteins incubated with ^{32}P-ATP alone (1 mM), in the presence of each of the designated nucleotides. Each value shown was derived from the binding in the presence of Mg + Na less that in the presence of Mg alone. Remaining constituents (other than the nucleotides) and volume of the incubating mixture were the same as has been described for Table 2.

uniformly accelerates the reaction while the addition of Na alone to the Mg-containing system stimulates the actitivy in some cases (16, 35, 36, 53, 54), but not in others (17, 49, 53, 55-62). The focus of the present report was on the catalyzed rate of labelling of the native and ouabain-treated microsomal phosphoproteins at 0°C, in the presence of Mg alone, in the presence of Mg + Na, and in the presence of Mg + Na + K. For comparative purposes, we elected to determine the overall rate of ATPase catalyzed hydrolysis at 0°C in the presence of Mg and of Mg + Na.

Figure 4 presents a plot of values of inorganic phosphate (P_i) liberated versus time of incubation at 0°C, in one representative experiment (out of 6 experiments) on a microsomal pellet, one aliquot of which was exposed to Mg alone, the other to Mg + Na. There was no apparent difference in the amount of P_i released from either aliquot at any time during the 10 minutes of incubation. The amount of P_i liberated after 10 minutes, 110 mμmoles/mgm protein, was approximately 5% of that liberated by incubation of the same microsomal system at 38°C (Shamoo, Y.E. and W.A. Brodsky - unpublished). Detection of such a small amount of P_i was made feasible by the use of a ^{32}-labelled ATP of high specific activity (10^4 cpm/mμmole of ATP). In comparison with the magnitude of ^{32}P-labelling of microsomal protein at 0°C., the amount of ATP hydrolyzed in 50 seconds at the same temperature, 10 mμmoles/mgm protein, was ca. 30 times greater than the average amount of Mg-dependent labelling, and ca. 13 times greater than the average of the (Mg + Na)-dependent labelling (see Tables 2, 3 and Fig. 4).

4. Time-Course of Phosphoprotein formation

a. Native microsomes

Even though the amount of ATP hydrolyzed in 50 seconds at 0°C is much greater than the amount of phosphoprotein formed, less than

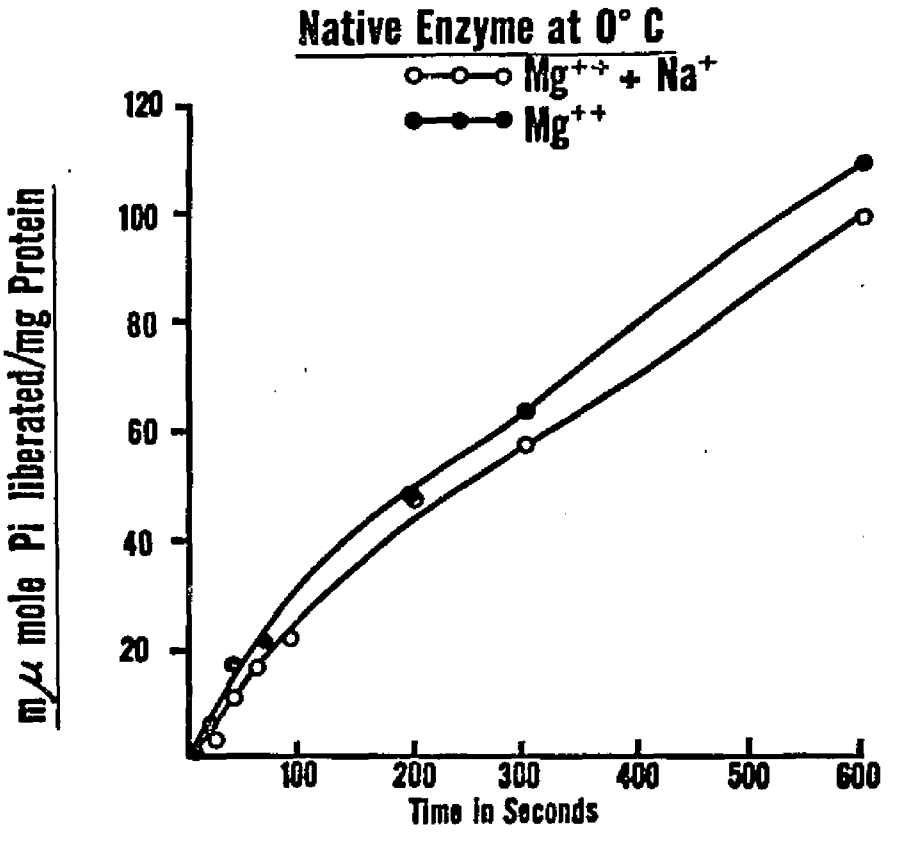

Fig. 4. Hydrolysis of ATP at 0°C in the absence of K. Amount of $^{32}P_i$ released versus time of incubation of native microsomal proteins with ^{32}P-ATP. Solid circles denote P_i release in the presence of Mg alone; and open circles, in the presence of Mg + Na. Apart from the absence of K, the remaining constituents and volume in each incubation flask was the same as has been described for Table 1.

5% of the available ATP (1 mM) was consumed during this period (see fig. 4).

In similarly designed experiments on crab nerve microsomes, Skou set the condition 25 μM ATP for almost complete hydrolysis of the available ATP at 37°C. During the hydrolysis, the (Mg + Na)-dependent ^{32}P-labelling of protein increased, reached a maximum in 10-20 seconds and then decreased during the next 30 seconds (14). We decided to design parallel experiments on ^{32}P-labelling as a function of time in bladder microsomes at 0°C where the phosphoprotein formation could be measured when the extent of ATP hydrolysis was negligible (as shown in fig. 4).

Native and ouabain-treated microsomes, prepared as described above, were incubated with ^{32}P-ATP in the presence of Mg alone and in the presence of Mg + Na. After terminating the reaction by PCA treatment, the amount of ^{32}P-labelling of protein and the amount of $^{32}P_i$ liberated were determined in each incubation flask -- as described in the Methods section. Since the results on overall hydrolysis (P_i liberated) at 0°C were essentially the same as those shown in fig. 4, they will not be displayed in the simultaneously paired results on ^{32}P-labelling.

Figure 5 is a plot of values of ^{32}P-labelled microsomal protein formed as a function of time of incubation at 0°C in one of four similar experiments on the native enzyme system.

Whereas, the order of magnitude of ^{32}P-labelling observed in the turtle bladder was similar to that of the Mg + Na dependent.

The order of magnitude of the ^{32}P-labelling of the native protein from turtle bladder was roughly similar to that reported for native microsomal protein from other tissues (14, 50, 57). The time-dependent pattern of the Mg-dependent labelling (see lower curve in fig. 5) was different from that found in crab nerve (14). The time-dependent pattern of the Mg + Na dependent labelling (see upper curve of fig. 5) was different from that found in kidney micro-

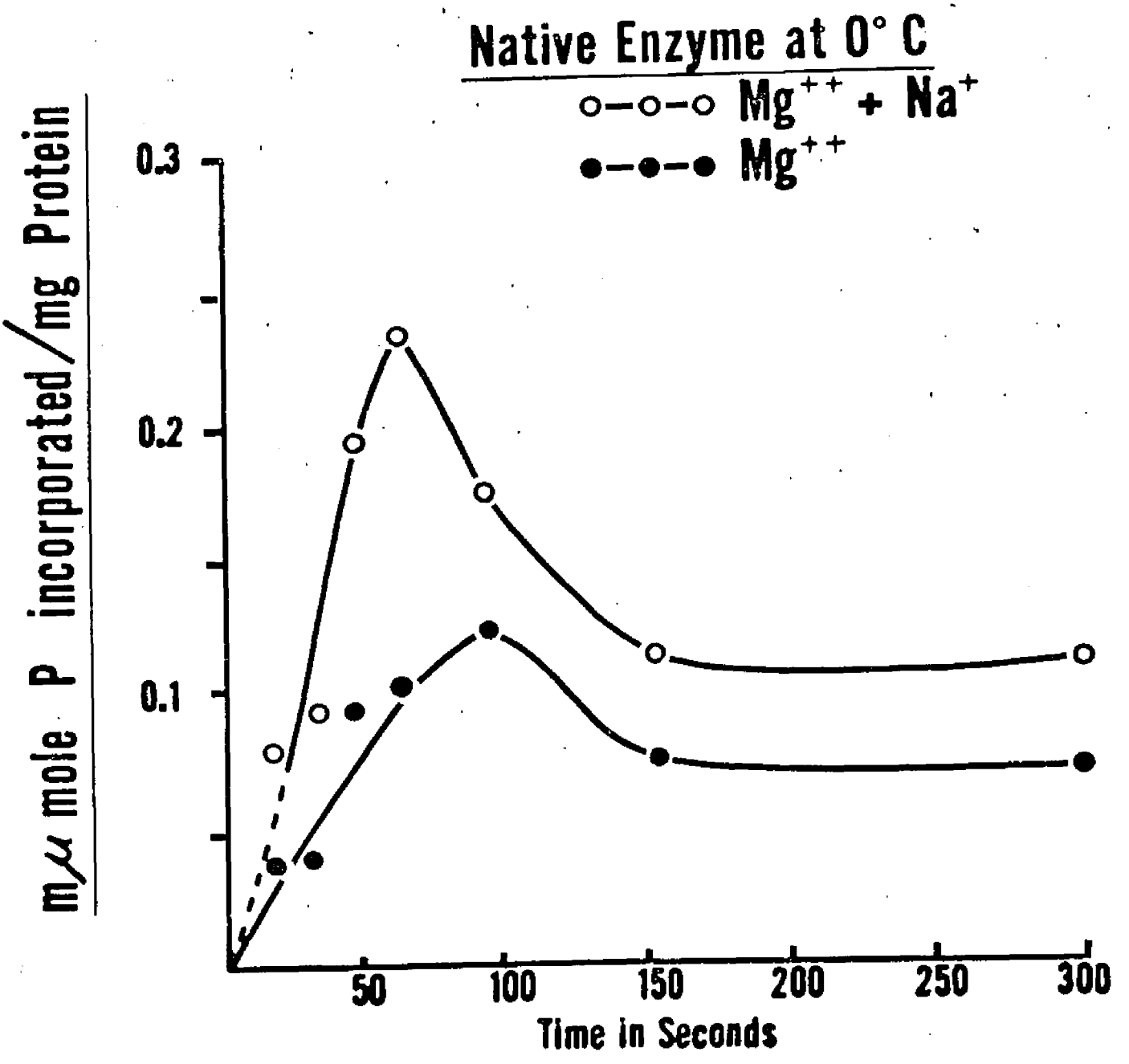

Fig. 5. Amount of ^{32}P-labelling of native microsomal proteins versus time of incubation with ^{32}P-ATP at 0°C. Solid circles denote labelling in the presence of Mg alone; and open circles, in the presence of Mg + Na.

Final concentrations of each constituent of the incubation mixture were: 85 mM Na, 15 mM K, 3 mM Mg, 3 mM γ-^{32}P-ATP (specific activity 10^9 cpm/μmole), 0.1 mM Tris-EDTA and 1 mM ouabain. Final volume 100 μl; temperature 0°C. Reaction was terminated by adding 100 μl 10% (w/v) of cold perchloric acid (final concentration 5%).

Each value of ^{32}P incorporated has been corrected for that incorporated by control microsomes which had been "killed" by PCA just prior to the incubation. The control or non-specific labelling amounted to 0.07 mμmoles/mg protein.

somes (37), but similar to that found in electric organ (50) and crab nerve (14). However, the similarity with that of crab nerve must be judged against the fact that a significant proportion of the ATP was consumed during the binding period in the experiments on crab nerve, but not in the experiments on turtle bladder.

b. Ouabain pre-treated microsomes

Figure 6 was constructed from the results of one of four experiments on ouabain treated microsomal proteins incubated at 0°C with ^{32}P-ATP. Data presented in the 2 plots are values of ^{32}P-ATP-labelled protein as a function of time of incubation.

The Mg + Na dependent labelling was greater than the Mg dependent labelling of the ouabain treated enzyme system throughout the 300 seconds of incubation, a pattern which was qualitatively similar to that shown in the previous figure on the time-dependent labelling of the native enzyme system. The ^{32}P-labelling under both conditions (Mg alone and Mg + Na) increased, reached maximal levels at 100 and 160 seconds respectively, and then decreased. The time required to reach maximal levels of ^{32}P-labelling in the presence of Mg alone in the ouabain treated system was the same as that in the native enzyme system -- ca. 100 seconds. However, the time required for maximal ^{32}P-labelling in the presence of Mg + Na in the ouabain treated system, 160 seconds, was greater than that in the native system, 60 seconds.

The post-maximal decrease in ^{32}P-labelling of the ouabain treated system was slower than that of the native system -- a retardation which was more obvious in the presence of Mg + Na than in the presence of Mg alone.

In summary, data figures 5 and 6 indicate that the effects of pre-treatment of the microsomal fraction with ouabain on the Mg + Na-dependent ^{32}P-labelling of the protein were: first, a "ouabain-shift" -- i.e. a prolongation of the time required to reach maximal levels of labelling;

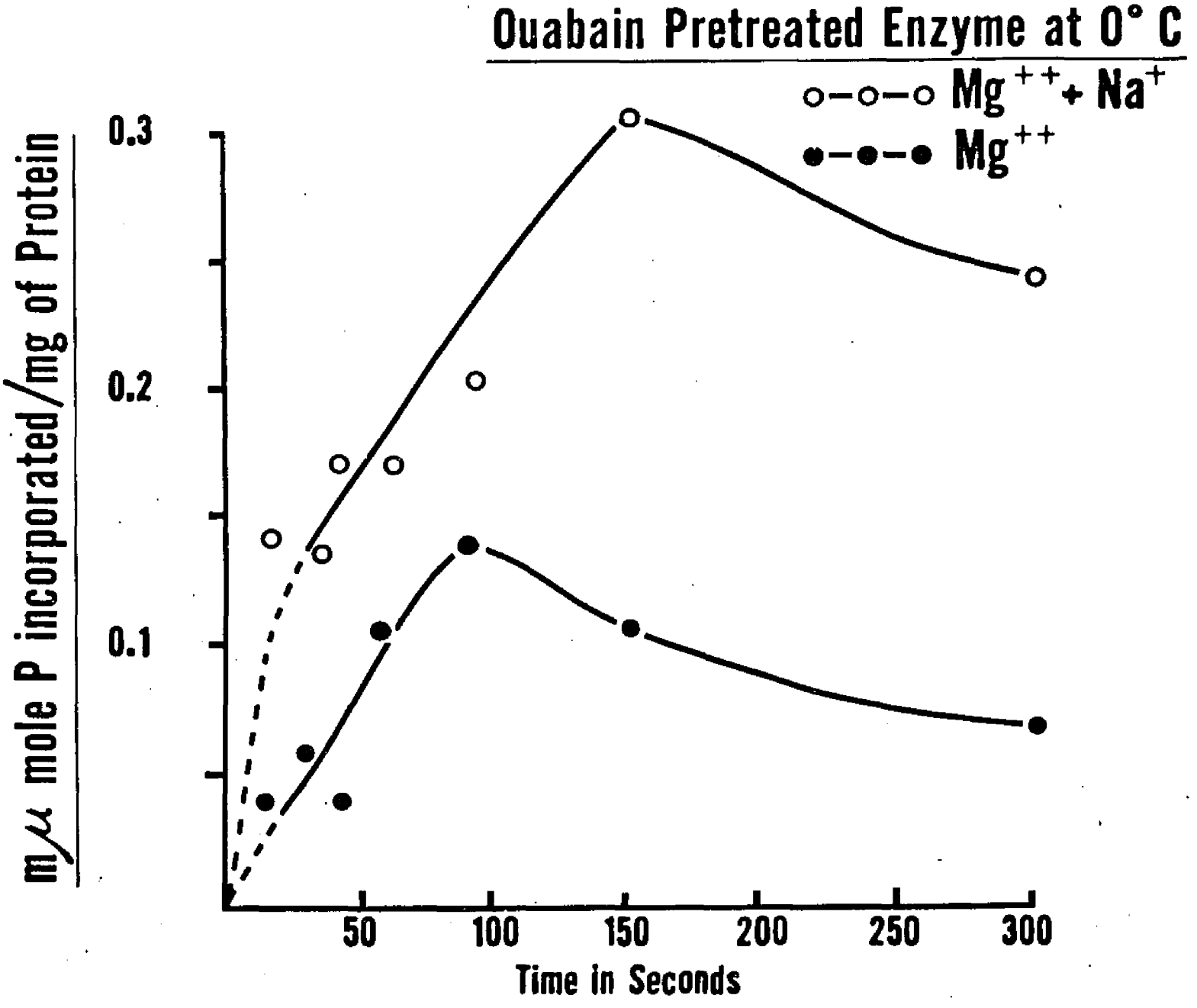

Fig. 6. Amount of ^{32}P-labelling of ouabain-treated microsomal proteins versus time of incubation with ^{32}P-ATP at 0°C. Solid circles denote labelling in the presence of Mg alone; and open circles, in the presence of Mg + Na. Except for ouabain, the composition and volume of all incubation mixtures were the same as those described for Fig. 3.

and second, a retardation in the rate of de-labelling of the phosphoprotein. Neither one of these effects was found in the Mg dependent labelling or de-labelling of the native protein.

5. Inorganic phosphate binding

Presumably, the ^{32}P label on the protein comes from ^{32}P-labelled ATP. However, some of the labelling might have come from the $^{32}P_i$ formed as the final product of the hydrolysis of ^{32}P-ATP. To test for the extent of $^{32}P_i$ binding, $^{32}P_i$-labelled inorganic phosphate, instead of ATP, was incubated against native and ouabain-treated microsomes.

Table 4 presents mean values and standard errors of P_i binding to the native and ouabain treated microsomal proteins in four experiments. The levels of P_i binding, 0.001-0.002 mμmoles/mgm Protein/50 sec. to the native protein and 0.007 to the ouabain-treated protein, were 1% and 3% respectively of the corresponding amount of the P binding from ATP. The extent of P_i binding was greater in the ouabain-treated than in the native protein -- a finding in accord with that recently reported by Siegel et al (58).

6. ^{14}C-labelling from ^{14}C-ATP

The next set of experiments, concerning the labelling of microsomal proteins from ^{14}C-ATP, was undertaken to obtain an independent check on the data of ^{32}P-labelling and to demonstrate directly, if possible, the presence or absence of an E-ATP complex in the microsomal proteins. Accordingly, ^{14}C-ATP, instead of ^{32}P-ATP, was the substrate incubated with the microsomal pellet under the standard conditions described in the Methods section.

Table 5 presents mean values for the absolute and relative labelling of native microsomal protein with ^{14}C after 50 seconds of incubation with ^{14}C-ATP at 0°C in six experiments.

Conditions for Labelling		Amount of Labelling ($m\mu$moles P_i/mgm/50 sec)
NATIVE	Mg^{++}	0.002 ± 0.002
	$Mg^{++} + Na^{+}$	0.001 ± 0.001
OUABAIN	Mg^{++}	0.007 ± 0.002
	$Mg^{++} + Na^{+}$	0.007 ± 0.002

Table 4. ^{32}P-labelling of native and ouabain-treated microsomal proteins incubated with inorganic phosphate ($^{32}P_i$), 5 mM. Remaining constituents (other than ^{32}P-ATP) and volume of the incubation mixtures were the same as has been described for Table 2. Each value shown is the mean and standard error of four experiments.

Conditions for Labelling	AMOUNT OF ^{14}C-ATP BINDING	
	Absolute (mμmoles/mg protein/50 sec)	Relative to Mg
Mg	0.72 ± 0.22	1.00
Mg + Na	0.39 ± 0.12	0.54
Statistical Parameters	Mean ± S.E. of the individual paired differences, $(Mg)_i - (Mg + Na)_i$	
	0.34 ± 0.11; $P < 0.02$ (n = 6)	

Table 5. ^{14}C-labelling of native microsomal proteins incubated with ^{14}C-ATP, 1mM. Remaining constituents (other than ^{32}P-ATP) and volume of the incubation mixtures were the same as has been described for Table 2.

Without exception, the addition of sodium to the Mg-containing incubation mixture resulted in a clean-cut decrease in the Mg-dependent ^{14}C labelling (from 0.72 to 0.39 mμmoles/mg/50 sec.) of the protein. The 46% decrease of ^{14}C-labelling in going from the Mg-containing to the Mg + Na containing incubation mixture was in striking contrast to the 158% increase of ^{32}P-labelling of the protein under the same conditions of incubation (see Table 5).

The findings on ^{14}C and ^{32}P-labelling of the microsomal proteins are consistent with the concept that the first intermediate between the enzyme protein (E) and substrate (ATP) is E-ATP (measured as the ^{14}C-labelled protein). Sodium induced acceleration of the intermediate steps in the hydrolysis could then result in an increased rate of accumulation of E~P (measured as an increase in ^{32}P-labelling of the protein) at the expense of the accumulation, but not at the expense of the rate of formation of E-ATP.

Verification of the findings on ^{14}C and ^{32}P-labelling was done as follows:

a. ^{32}P-ATP and ^{14}C-ATP were incubated together with cold ATP, 1 mM, in one flask with the microsomal mixture described in the Methods section and used throughout this work. Preliminary data from two experiments showed that the Mg-dependent binding of ^{14}C was greater than that of ^{32}P; that the addition of Na decreased ^{14}C binding (so that the ^{14}C activity ratio, (Mg + Na) / (Mg)$\approx$0.5) and simultaneously increased ^{32}P binding (so that the ^{32}P activity ratio (Mg + Na / (Mg)$\approx$2.2). All three findings with the simultaneously added isotopic labels were about the same as those found with individually added isotopic labels (see Tables 2 and 3). Although this matching sounds reasonable, the reason for the excess of ^{14}C over ^{32}P binding in the Mg-dependent reaction is not

intuitively obvious from these data.

b. In the next two experiments on ^{14}C-labelling of the protein, the reaction was terminated solely by rapid filtration of the incubation mixture. No PCA was used to 'kill' the reaction in the flask or to wash the microsomal pellet remaining on the filter paper. The ^{14}C-labelling pattern of this nearly-native microsomal pellet with Mg alone and with Mg+Na was essentially the same in absolute magnitude and degree of Na-induced decrement as that found in the PCA treated microsomal pellet. Apparently the ^{14}C-labelled protein is acid-stable.

Parenthetically, the ^{14}C-ATP, ^{32}P-ATP, and cold ATP were found to be chromatographically identical.

C. Exchange reaction

1. Factors Affecting the Exchange Rate

a. Amount of enzyme protein. Figure 7 presents two composite plots of values on the rate of conversion of ^{14}C-ADP to ^{14}C-ATP as a function of the amount of microsomal proteins. The rate of the exchange was expressed in terms of the amount of ^{14}C-ATP formed as a percentage of the initial amount of ^{14}C-ADP. Each point on each of the curves is the mean of four determinations from four separate experiments on the same batch of microsomes.

The lower curve shows the experimentally determined values for the exchange rate. The upper curve shows the values after correcting for the hydrolytic loss of the ^{14}C-ATP formed during the exchange reaction. This hydrolytic loss, presumably due to the action of the Mg-dependent ATPase, was 10 micromoles/mg of microsomal protein/hour, on the average, as determined in a separate set of experiments on the same microsomes.

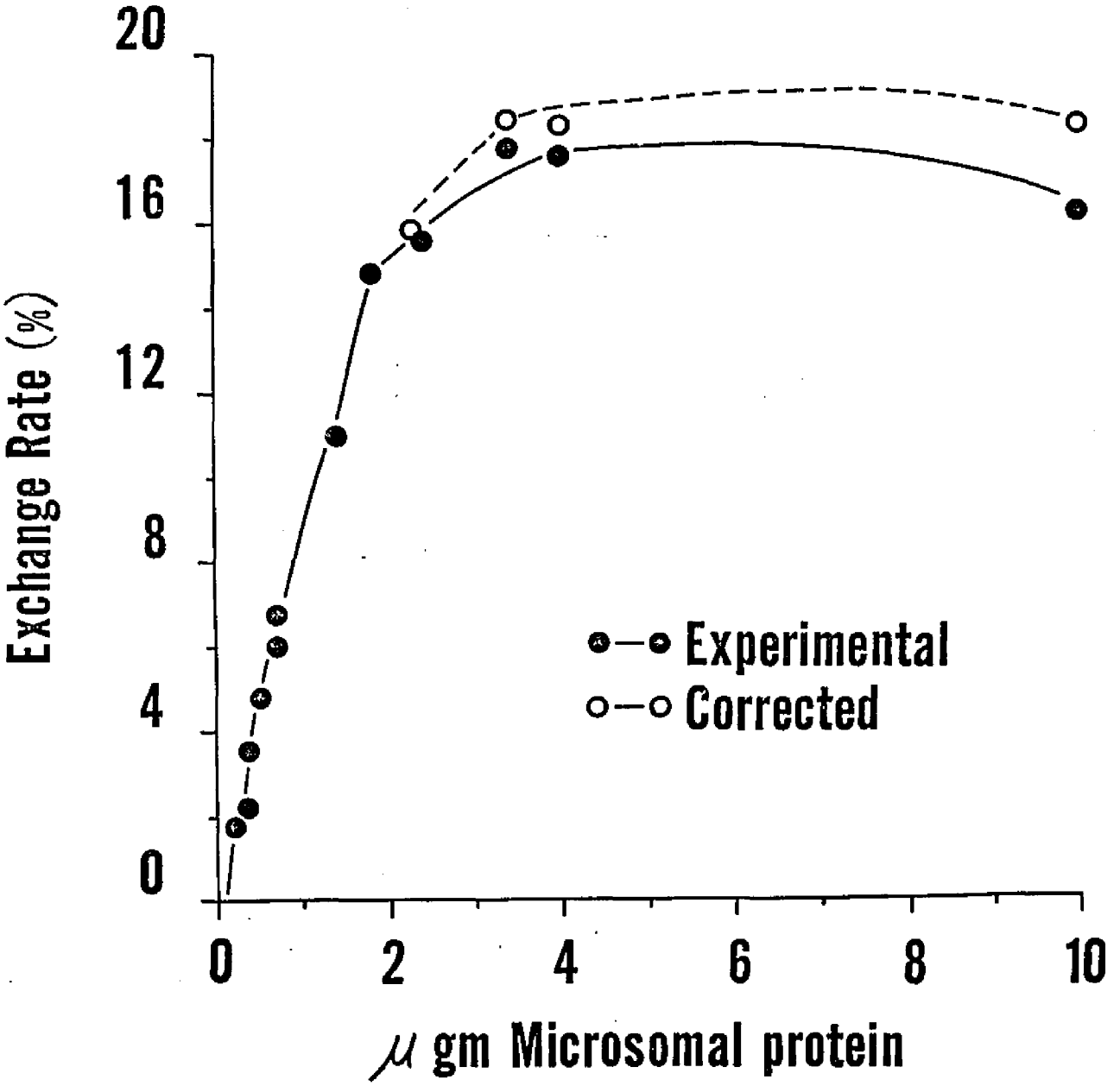

Fig. 7. Exchange rate as a function of the amount of microsomal protein. Each point is the mean value obtained from 4 experiments on a single batch of microsomes. Exchange rate is defined as the amount of ^{14}C-ATP formed in 10 minutes at 38°C as percentage of the initial amount of ^{14}C-ADP. The standard errors are represented by the vertical bars.

Whenever the radius of the circle was equal to or greater than the SE, the vertical bars are not shown in the graph. (This definition of the exchange rate and the graphic presentation of the mean value and its SE will be the same throughout the remainder of this report.)

Appropriate dilutions of the enzyme were added to each reaction mixture, and the subsequent incubation and assay were as described in the Methods section. Each tube contained 3 mM $MgCl_2$, 5 mM ATP, 1.6 mM ^{14}C-ADP, 0.1 mM Tris-EDTA, 40 mM Tris-HCl (pH 7.3) as buffer, and varying amounts of microsomal protein as indicated along the abscissa in a total volume of 30 μl. The solid circles represent experimentally observed results. The open circles were the corresponding corrected values of experimentally observed results for the hydrolytic loss of the ^{14}C-ATP formed during the exchange reaction.

Even without correcting for hydrolytic losses of ^{14}C-ATP, as much as 17% of the ^{14}C-label of ADP was incorporated into ATP during a 10 minute period of incubation at 38°C. The corrected and uncorrected values do not differ appreciably in the presence of 4 μgm or less of microsomal protein. However, an appreciable correction to the exchange rate is required in the presence of 5 or more μgm of microsomal protein. This type of correction is more noticeable in turtle bladder microsomes than in other tissues because of the relatively high Mg-dependent ATPase activity -- which accounted for the hydrolysis of 10% of the total ATP in the 10 minutes of incubation with maximal amounts of microsomal proteins (10 μgm) used for the experiments on the exchange reaction.

The pattern of corrected values versus amounts of enzyme resembles that expected of an exchange reaction (48), whereas the pattern of raw values versus amount of enzyme (particularly in the presence of high concentrations of microsomal protein) deviates somewhate from that expected of a pure exchange reaction.

b. Concentration of ATP and ADP. In the next set of experiments, the amount of microsomal protein (ca. 2 μgms) and the nucleotide concentration ratio, [ATP]/[ADP] = 4.0 were fixed, thereby permitting observations on the exchange rate as a function of the concentration of ATP.

Figure 8 is a composite plot of values of the catalyzed rate of the ADP-ATP exchange reaction as a function of the concentration of ATP in the presence of Mg (upper curve), and in the presence of Mg + Na (lower curve). Each point on each of the curves is the mean of four determinations from four separate experiments on the same batch of microsomes.

The most obvious finding is that the catalyzed rate of exchange in the presence of Mg was significantly greater than that in the presence

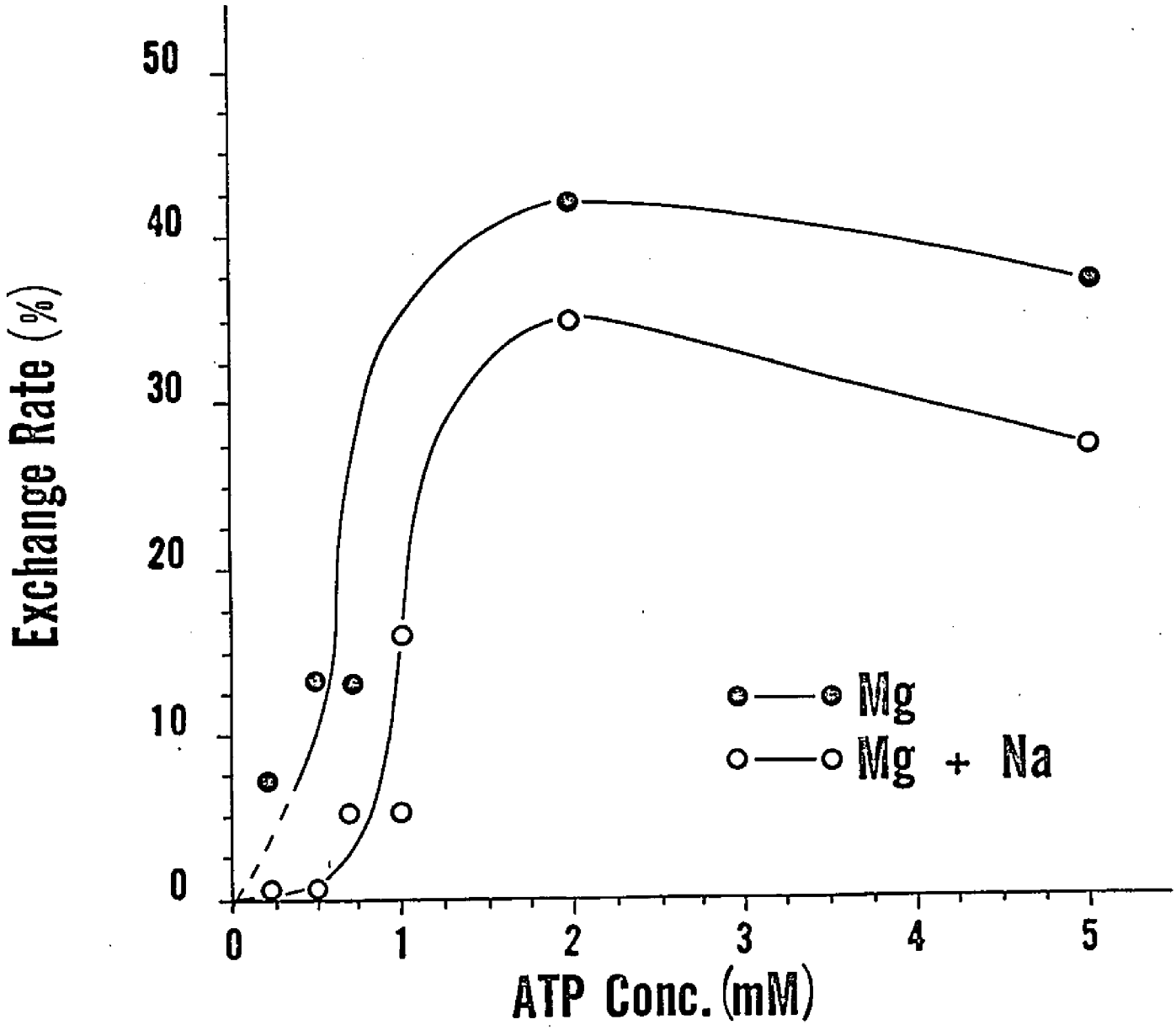

Fig. 8. Exchange rate versus ATP concentration; ATP/ADP ratio was kept constant (4/1). Solid circles indicate the presence of 3 mM $MgCl_2$; open circles indicate the presence of 3 mM $MgCl_2$ + 85 mM NaCl; amount of microsomal protein in each vessel was 5 µgm. All other conditions and ion concentrations were as described for Fig. 7 and in the Methods section.

The geometric center of each point is the mean value of the exchange found in 4 experiments on a single batch of microsomes, and the radius of each circle is the maximal standard error as described for fig. 7.

of Mg + Na over the entire range of ATP concentrations (0.2 - 5.0 mM) used. The Na-induced decrement in the exchange rate, reminiscent of the previously reported Na-induced decrement in ^{14}C-ATP binding on the microsomes, is conistent with the reaction model postulated (see Fig.2) Thus, with no sodium present, the exchange reaction proceeds through both reaction steps 1 - 2 and 1 - 4, by the following steps:

$$1)\ E + ATP \underset{}{\overset{Mg}{\rightleftharpoons}} E\text{-}ATP$$

$$2)\ E\text{-}ATP \overset{Mg}{\rightleftharpoons} E\sim P + ADP$$

and

$$4)\ E\text{-}ATP \underset{Na}{\overset{Mg}{\rightleftharpoons}} E'\sim P + ADP$$

where transphosphorylation can be accomplished through the two reversible reactions. When Na is added, the irreversible reaction 5,

$$5)\ E'\sim P \xrightarrow{Mg,Na} E'\text{-}P,$$

is accelerated, which provides a sink for E'$\sim$P thereby reducing the extent of the reversible transphosphorylation of ADP to ATP via reactions 1 and 4.

The early report showing a sodium-induced decrement of ^{14}C-binding to microsomes, (see Table 5) could be explained by postulating the site of sodium action on reaction 4 -- which would predict a sodium-induced increase of transphosphorylation. However, Figure 8 shows a clear-cut sodium-induced decrease in transphosphorylation, which excluded reaction 4 as the sole site of sodium action. The current postulate that sodium acts on reaction 5, is fully consistent with the sodium-induced decrease in the exchange rate (Fig.8) as well as with the sodium-induced decrease in binding of ^{14}C-ATP to the microsomes (Table 5).

The functional form of the plot shown in Figure 8 is somewhat complex. At low concentrations of ATP, 0.2 to 1.0 mM, the rate function

resembled that expected of cooperative homotropic kinetics (60); and at high concentrations of ATP, 2.2-5.0 mM, the rate function resembled that expected of substrate inhibition at or near the saturation range of the reaction.

c. pH. Figure 9 presents two composite plots of values on the rate of exchange in the presence of Mg, and in the presence of Mg + Na versus pH. Each point is the mean value of four determinations from four individual experiments on a single pool of microsomes.

Neither the Mg nor the Mg + Na dependent exchange rate showed any marked change due to variation of the pH from 5.5 to 9.0. The sodium-induced decrement of the exchange rate was readily detectable over the entire pH range.

The pH independence and the sodium-induced decrement of the exchange rate in turtle bladder microsomes differ in certain respects from the corresponding data obtained from microsomes of rat brain (51) and electric organ of the eel (40). In the rat brain preparations (51), as in the turtle bladder, the Mg-dependent exchange rate reached maximal levels over a broad range of pH. On the other hand, in contrast to the turtle bladder, the sodium-stimulated exchange rate reached its peak value at a pH of 7.5 microsomes of the rat brain (51), and at a pH of 8.0 in microsomes of the electric organ of eels (40).

d. Concentration of magnesium. Figure 10 presents two composite plots of values on the rate of the exchange reaction as a function of Mg concentrations in the presence of Na (lower curve), and in the absence of Na (upper curve). In both cases (with and without Na), the kinetic pattern of the exchange rate was that of a Michaelis-like function. At low concentrations of Mg (0.0 to 0.6 mM) there is no marked sodium-induced stimulation -- as has been observed for the electric eel microsomes

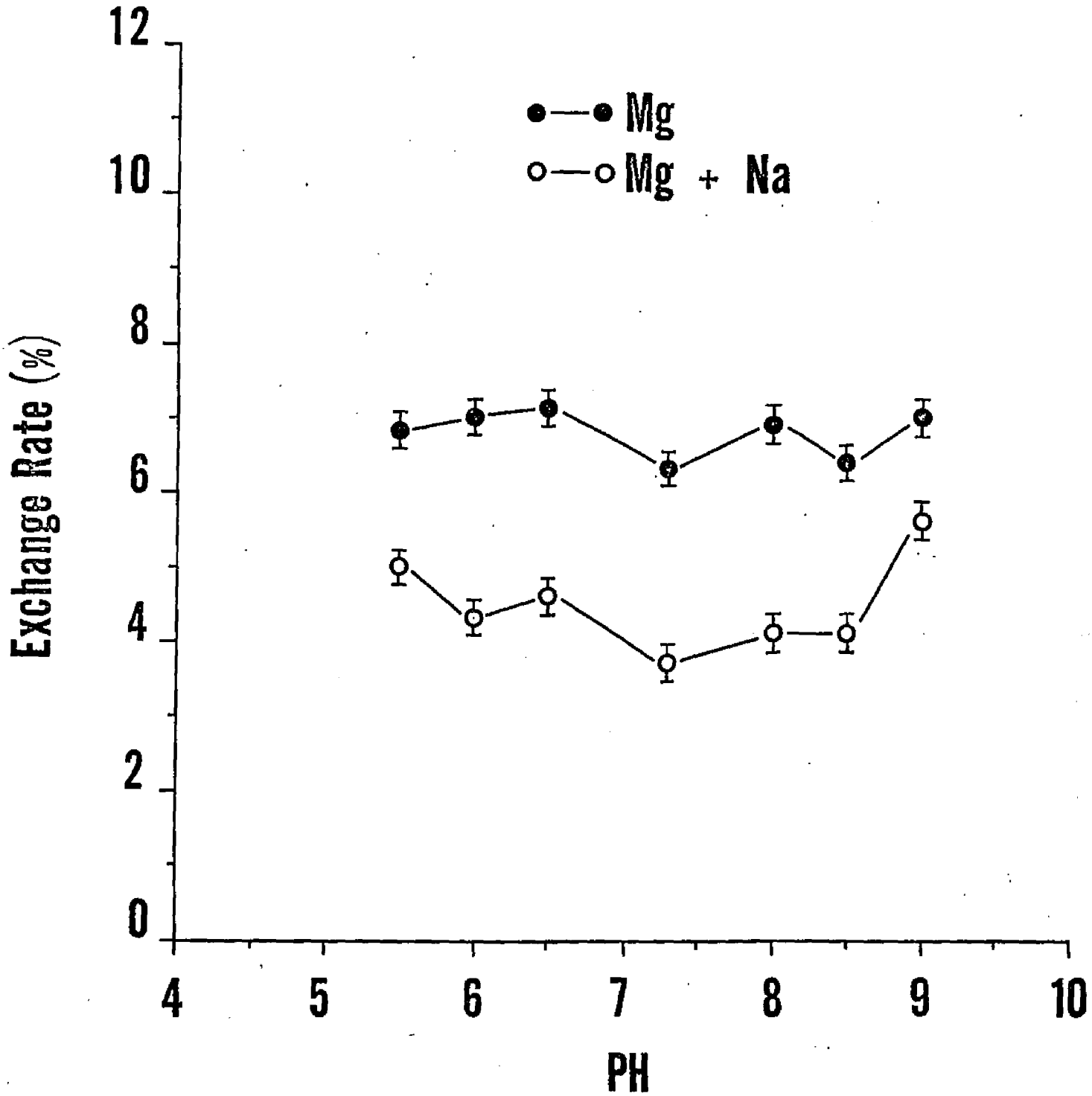

Fig. 9. Exchange rate versus pH. The geometric centers of the circles is the mean value of the exchange (n=4) and the vertical bars denote the magnitude of the standard error. Solid circles represent values in the presence of Mg; open circles represent values in the presence of Mg + Na. Each tube contained 3 mM $MgCl_2$, 5 mM ATP, 1.6 mM ^{14}C-ADP, 0.1 mM Tris-EDTA, 40 mM Tris-HCl(pH 7.3) as buffer, 5 µgm of microsomal protein in a total volume of 30 µl; sodium when present was 85 mM. All other conditions were as described in the Methods section. The definition of exchange and the statistical basis for each point are as indicated in the legend of figure 7.

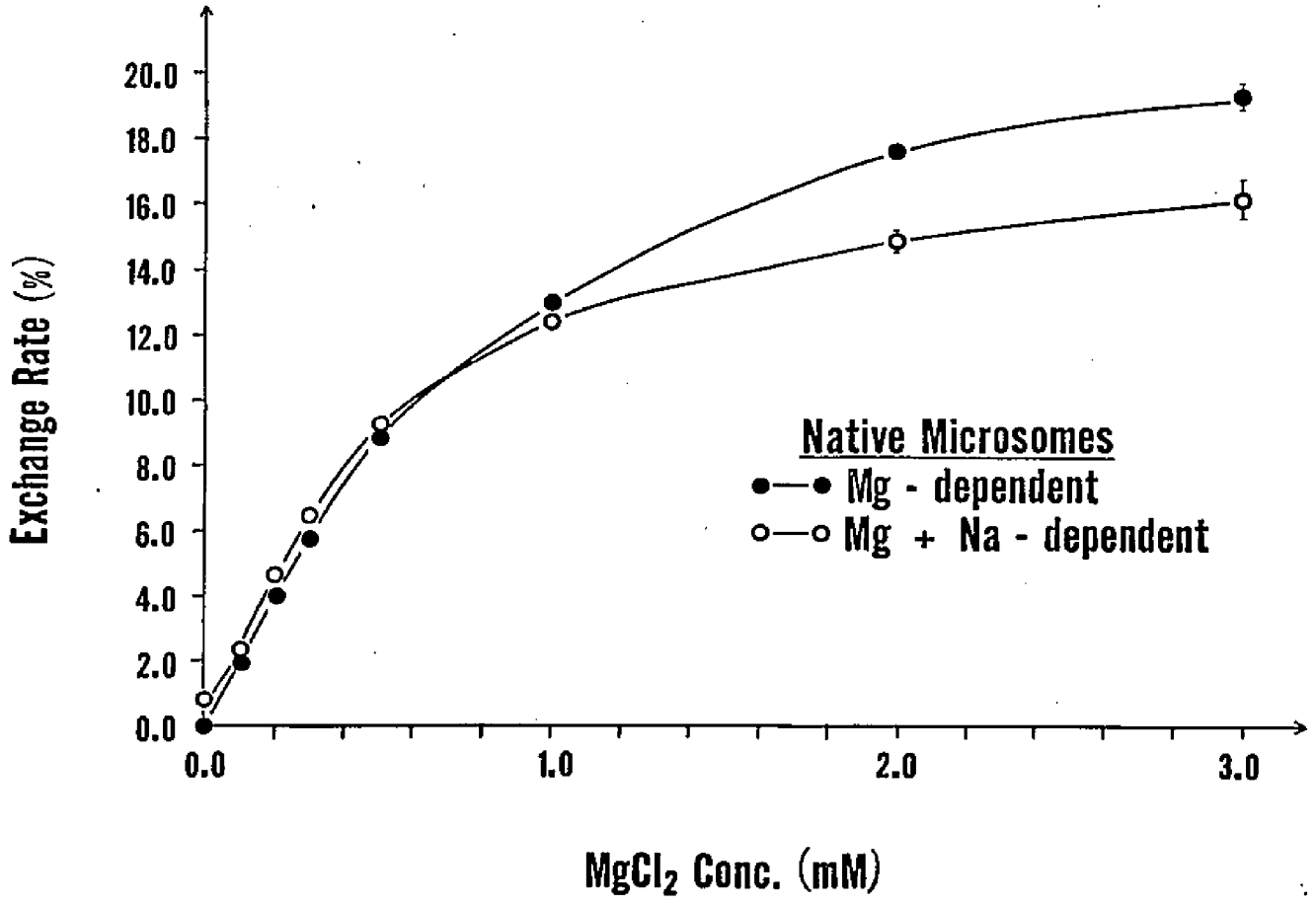

Fig. 10. Exchange rate versus Mg concentrations. The geometric centers of the circles denote mean values (n=4); the radius denotes the maximal standard error; and the vertical bars denote the standard error when the magnitude exceeds that of the radius. Solid circles represent values in the presence of Mg; open circles represent values in the presence of Mg + Na. Each tube contained 5 mM ATP, 1.6 mM ^{14}C-ADP, 0.1 mM Tris-EDTA, 40 mM Tris-HCl (pH 7.3), 5 µgm of microsomal protein in a total volume of 30 µl, Mg concentrations as denoted. Sodium when present was 85 mM. Incubation and assay procedures were as described in the Methods section. The definition of exchange rate is that stated for fig. 7.

(51). At high concentrations of Mg (0.6 to 3.0 mM), the presence of Na resulted in a significant decrease in the exchange rate, as has been observed by others (51,61) in different tissues.

The aforementioned kinetic patterns are consistent with reactions 1, 2, 4, and 5 of the scheme presented in Fig.2. With Mg alone, the exchange is mediated through reactions 1 and 2 of the Mg-dependent ATPase sequence, and simultaneously through reactions 1 and 4 of the Na, K dependent ATPase sequence. With Mg + Na, the exchange is mediated through the same reactions, but the Na-induced sink for E'$\sim$P reduces effective transphosphorylation of ADP by the E'$\sim$P.

The Mg-dependent reaction sequence terminates in reaction 3,

$$3)\ E\sim P \longrightarrow E + P_i,$$

which may or may not require Mg.

e. Concentration of sodium. Figure 11 presents a composite plot of values on the exchange rate as a function of sodium concentrations. The increase in sodium concentration resulted in a marked decrease in the Mg-dependent exchange rate. For example, the exchange rate approached zero when the Na concentration was increased to 120mM.

Such a sodium-induced decrease in the exchange rate is consistent with the data of Figure 10 on exchange activity versus Mg in the presence of Na; and is also consistent with the postulate that sodium acts mainly on reaction 5 in the model reaction system, and supports the contention that reaction 5 is a nearly irreversible step.

However, the fact that Na, at 120 mM, reduced the exchange to near-zero levels -- without a simultaneous change in the overall hydrolysis suggests: (a) that in addition to its effect on E'$\sim$P, sodium changes the internal energy state of E$\sim$P such that it can no

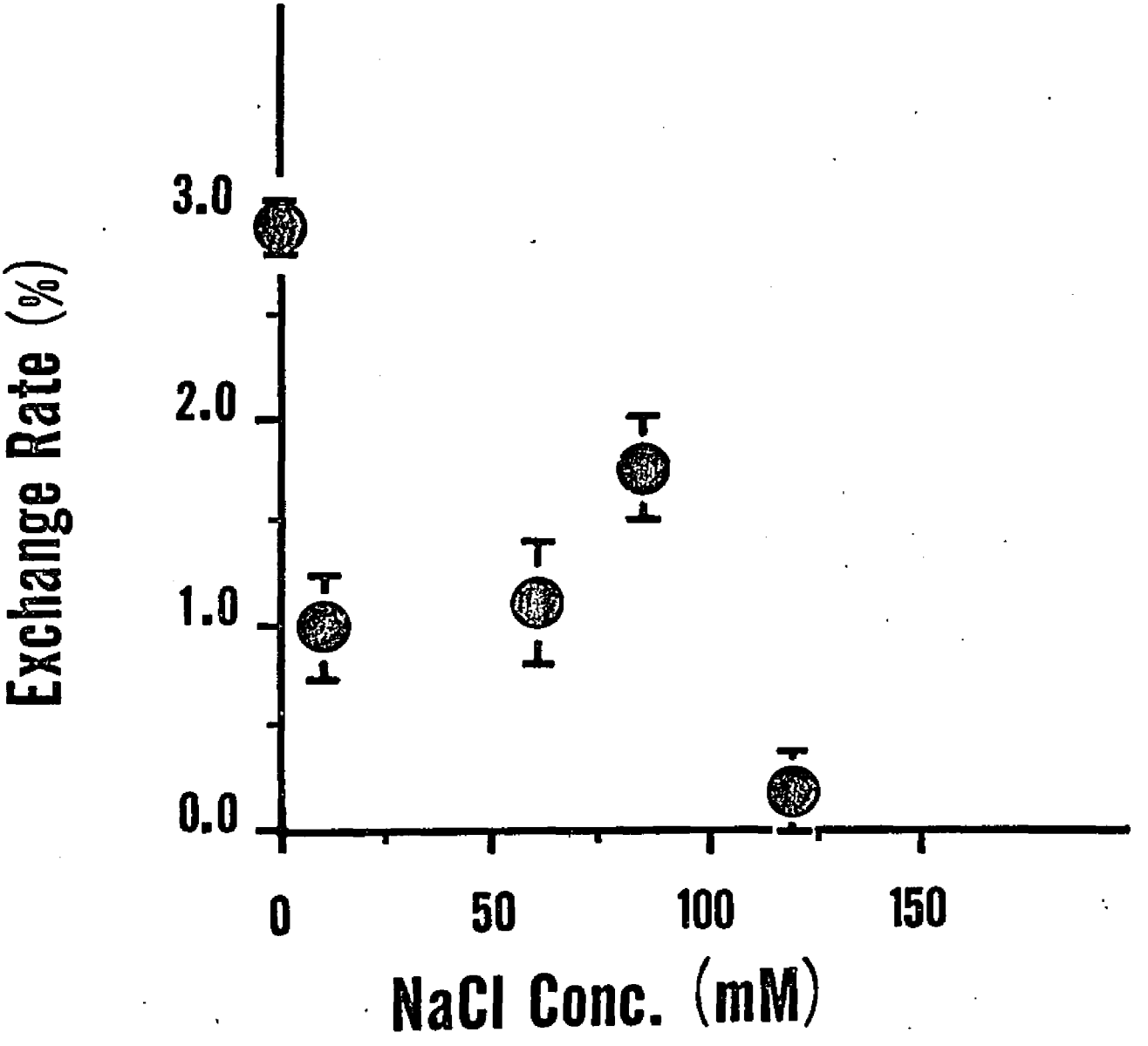

Fig. 11. Exchange rate versus Na concentrations. The geometric centers (mean values, n=4) and the vertical bars (±SE) are the same as described for Fig. 9. 0.1 mM $MgCl_2$ was used. Sodium as denoted. The rest of ionic concentrations and conditions were as described for Fig. 10.

longer phosphorylate the ADP, but can continue to degrade into E and P_i; or (b) that reaction 2 was an irreversible step with E-P formation -- which would imply that a "pure" Mg-dependent, Na-independent exchange reaction does not exist in the microsomal proteins of turtle bladder.

f. Potassium. The effect of 15 mM K on the exchange rate was examined in 2 experiments on a single batch of microsomal proteins. The conditions for incubation and the composition of the reaction mixture were the same (except for K additions) as has been described herein. The mean values of each exchange rate, expressed as percentages of that obtained with Mg alone (raw value for Mg = 15%), were as follows: Mg, 100; K alone, 3; Mg + K, 100; Mg + Na, 68; Mg + Na + K, 65. In other words, the addition of K in the presence of Mg and Mg + Na had no detectable effect on the measured exchange rate.

The data on Na + K dependent ATPase and K-induced stripping of ^{32}P from the phosphoprotein (See Tables 2 and 3) are consistent with placing the site of the K effects at reaction 6,

$$6)\ E'\text{-}P \xrightarrow{K} E' + P_i$$

This, in turn, is particularly consistent with the lack of any K-induced effect on the exchange reaction, thus suggesting that the rate constant for reaction 6 is less than that of reaction 5.

2. Effect of Inhibitors on the Exchange Rate

Table 6, a summary of results on a single batch of microsomes, presents values of relative activity of the exchange rate (columns 1 and 2) and of the overall hydrolysis (columns 3 and 4) in the presence of three inhibitors under the specified ionic conditions. Values of the exchange rate are expressed as percentages of the native Mg-dependent activity; and those of hydrolysis (ATPase activity), as percentages of

Conditions	Exchange Rate		ATPase activity	
	Percent of Mg-dependent		% of control	% of control
	Mg	Mg + Na	Mg	Na + K
Native	100	83 ± 2	100	100
Ouabain ($1X10^{-3}M$) pre-treated	145 ± 2	132 ± 2	105 ± 3	4 ± 2
NEM ($1X10^{-3}M$) pre-treated	16 ± 9	31 ± 5	92 ± 3	55 ± 3
oligomycin (6.8µgm/ml)	111 ± 4	96 ± 12	90 ± 2	50 ± 5

Table 6. Exchange rate and ATPase activity of the native and of the inhibitor-treated microsomal protein. Exchange rates were normalized with respect to the Mg-dependent activity of the protein. ATPase activities were normalized with respect to the Mg-dependent activity of the native protein, or with respect to the Na + K-dependent activity of the native protein. Each reaction tube contained: 3 mM $MgCl_2$, and when indicated, 85 mM NaCl and 15 mM KCl. Other ion concentrations and conditions were as described for Fig. 4. Na + K ATPase activity and exchange rate were determined as described in the Methods section.

the native Mg-dependent or of the native Na + K dependent activity. Apart from 14 separate determinations on the native microsomes, the mean values and standard errors were estimated from the data of four separate experiments on a single pool of microsomal pellets, one aliquot of which was treated with ouabain, one with NEM and one with oligomycin.

In the native microsomal pellet (first row), the exchange rate in the presence of Mg + Na was 83% of that in the presence of Mg alone ($P < 0.001$). The native Mg-dependent exchange rate was 12 micromoles/mg protein/hour. Concomitantly, the hydrolytic activity of the same pellet (rate of P_i released), was 10 micromoles/mg protein/hr with Mg, and 21 micromoles/mg protein/hr with Mg + Na + K.

In the inhibitor-treated aliquots of the same microsomal pellet, the exchange rate was: (a) increased in the presence of ouabain ($P < 0.001$) which completely inhibited the Na + K ATPase activity; (b) decreased in the presence of NEM ($P < 0.01$) which inhibited 45% of the Na + K ATPase activity; and (c) hardly changed in the presence of oligomycin ($0.05 < P < 0.1$) which inhibited 50% of the Na + K ATPase activity.

Next we performed a series of experiments on the ADP:ATP exchange rate in the inhibitor-treated microsomes as a function of the Mg-concentration.

a. Ouabain. Figure 12 presents two composite plots on values of the rate of exchange reaction in ouabain-treated (10^{-3} M) microsomes as a function of the Mg concentration without Na (upper curve), and with Na (lower curve).

The maximal rates of exchange observed in the ouabain-treated microsomes, 25% with Mg alone and 22% with Mg + Na, were greater than the corresponding rates (19 and 15% respectively) in the native microsomes. The ouabain-induced acceleration of the Mg-dependent ADP:ATP exchange rate in turtle bladder microsomes contrasted with the lack of change

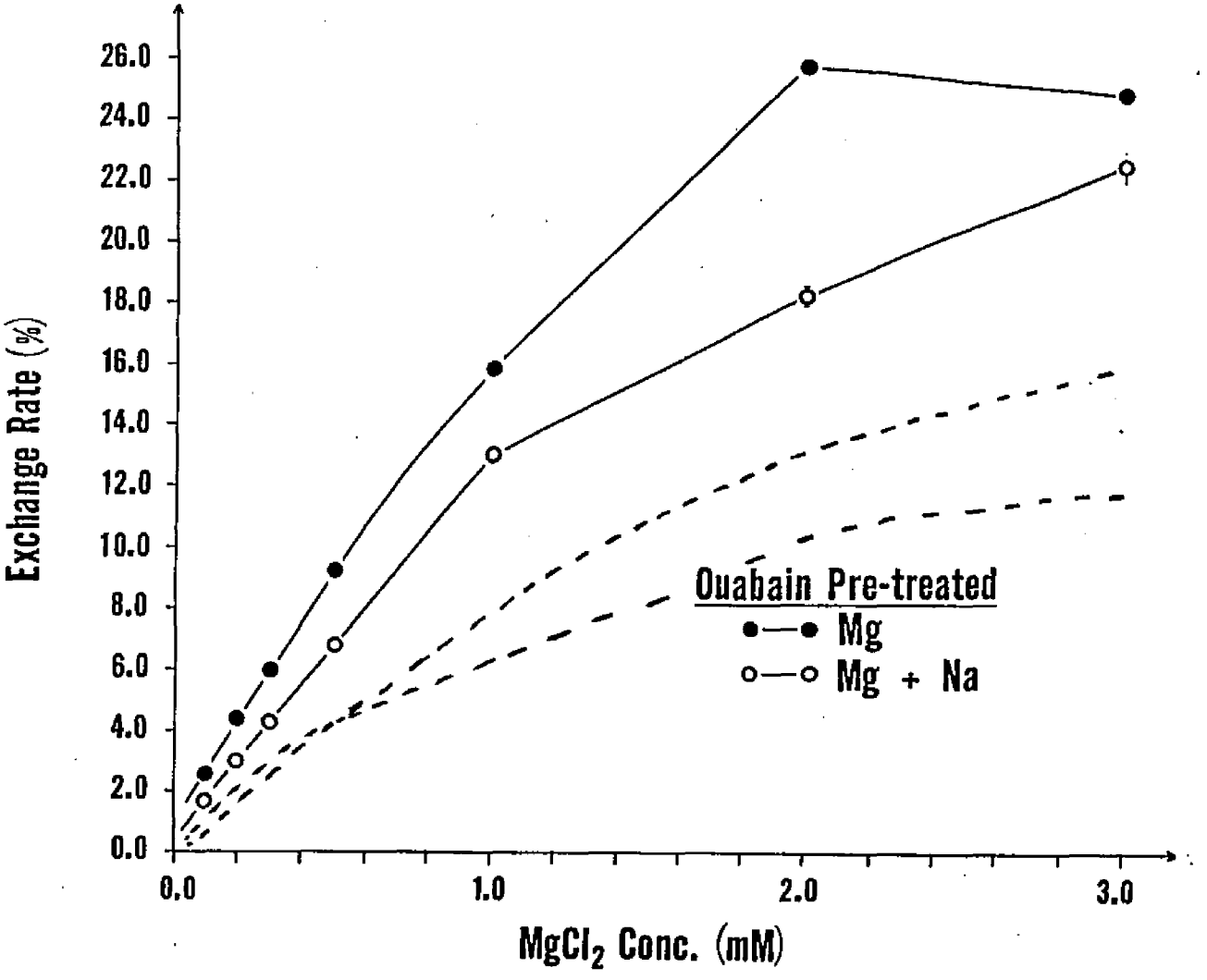

Fig. 12. Exchange rate in microsomes pre-treated with ouabain, (10^{-3}M). All ion concentrations and conditions were as described for fig. 10.

The dotted lines are the schematic presentations for the Mg-dependent exchange (upper curve) and Mg + Na-dependent exchange (lower curve) in the native microsomal protein (see fig. 10). The geometric centers (mean values, n=4) and the vertical bars (±SE) are the same as described for fig. 9.

reported for the Mg-dependent exchange rate in microsomes of the electric eel (40).

The figure also shows that sodium retarded the exchange rate in the ouabain-treated microsomes over the entire range of Mg concentration used (0.1 - 3.0 mM). In the native microsomes, sodium failed to retard the exchange rate in the presence of low Mg concentrations (0.1 - 0.6 mM), but did retard the exchange rate at high Mg concentrations (1.0 - 3.0 mM).

Several investigators (6,14,62,63), have suggested that ouabain inhibits the Na + K ATPase by interacting at the K-site of the enzyme (reaction 6). Whereas the ouabain-induced acceleration of the exchange rate in turtle bladder microsomes cannot be explained by the contention that ouabain acts solely on the K-site (reaction 6) of the enzyme, such an observation is consistent with the postulate that ouabain interacts at site of reaction 5 either solely or in addition to its interaction at the site of reaction 6. Inhibition at the site of reaction 5 would slow down the rate of conversion of E$\sim$P to E'-P, thus allowing a greater exchange rate.

This new postulate on the site of ouabain action is consistent with other observations on the exchange rate and on ^{32}P-binding in the presence of ouabain. For example, the sodium-induced decrease in the exchange rate (Figure 8) could be due to some unspecified type of interaction between sodium and ouabain with an occupation site on the protein; and the previously reported sodium-induced increase in ^{32}P-binding (See Tables 2 and 3, Figures 3,4 and 5) could be due to the interaction of sodium with the protein site involved in reaction 5, as well as to a minor interaction of sodium with the protein site involved in reaction 4.

b. NEM. Figure 13 presents two composite plots of values on the rate of the exchange reaction of NEM-treated (10^{-3} M), microsomes as a function of Mg concentration, without Na (lower curve) and with Na (upper curve).

Apparently NEM, at 10^{-3} M, caused a large decrease in both exchange rates -- that in presence of Mg and that in presence of Mg + Na. (See also Table 6). Despite this inhibition, the addition of sodium was associated with a small increase in the exchange rate of the NEM-poisoned enzyme system. The results are consistent with the model reaction scheme, if one postulates that NEM blocks the reaction at sites 4 and 5.

c. Oligomycin. Table 7 presents mean values ± SE of the catalyzed exchange rates in microsomes treated with progressively increasing concentrations of oligomycin. The range of concentration used was similar to that used by others in similar studies on other tissues (40,51). The first column denotes the concentration of oligomycin; the second and third columns, the values of Mg and Mg + Na dependent exchange rates respectively.

Increases in the concentration of oligomycin resulted in little or no increase in the exchange rate. Addition of Na caused a slight decrease in the exchange rate in the native and in the oligomycin-treated microsomal preparations.

No conclusions were made with respect to the effect of oligomycin on the model scheme presented in the introduction section, because the oligomycin effects were little or nothing under the present conditions.

3. Substrate Specificity

In order to determine the nucleotide specificity of the exchange rate, experiments were performed in the presence of equimolar concentrations (5mM) of ITP, CTP, GTP, UTP, instead of ATP. Ionic conditions in all

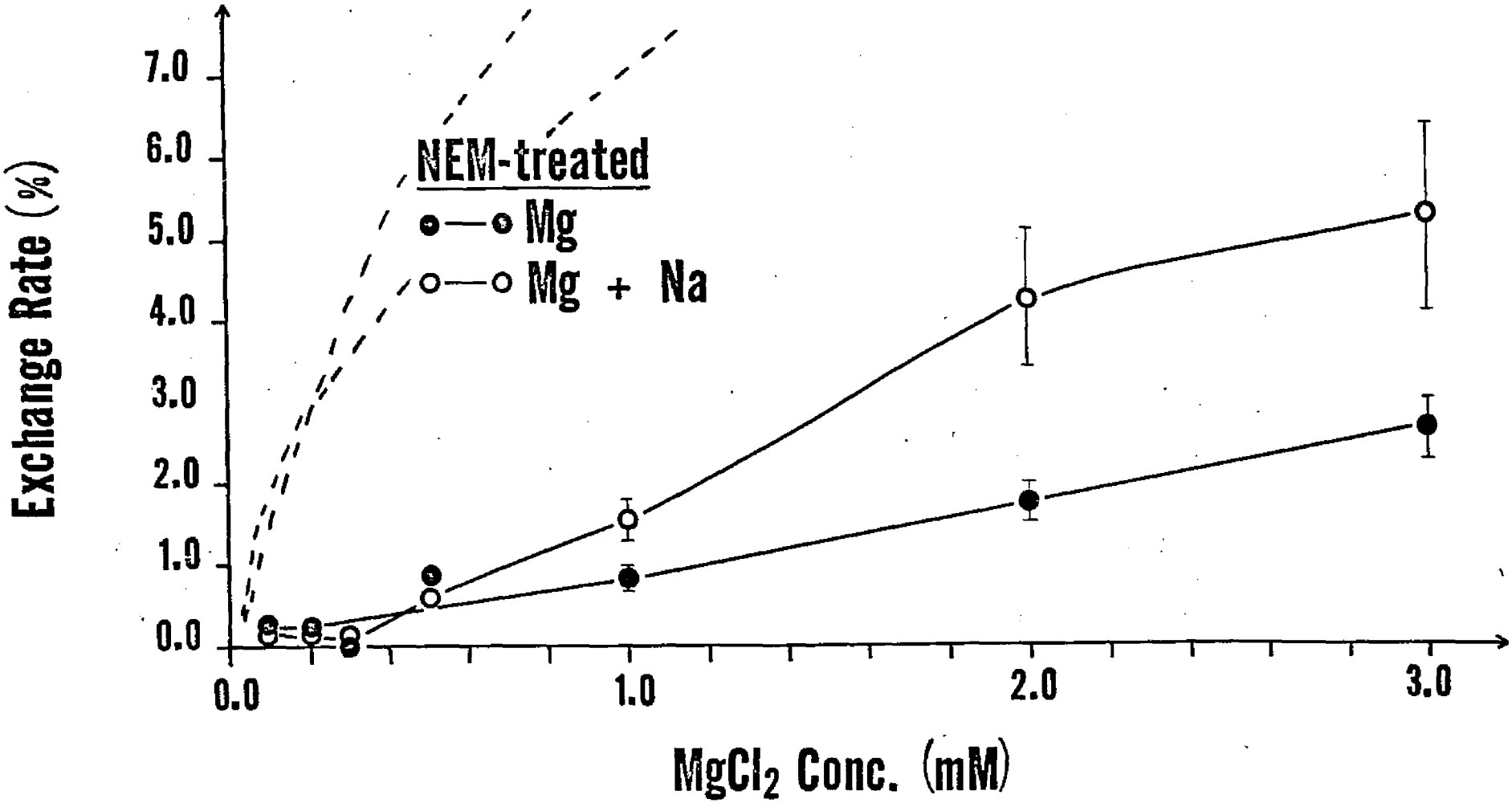

Fig. 13. Exchange rate in microsomes pre-treated with NEM, (10^{-3}M). All other ion concentrations and conditions were as described for fig. 10.

The dotted lines are the schematic presentations for the Mg-dependent exchange (upper curve) and Mg + Na-dependent exchange (lower curve) in the native microsomal protein (see fig. 10). The geometric centers (mean values, n=4) and the vertical bars (±SE) are the same as described for fig. 9.

Conc. of Oligomycin µgm/ml.	Exchange rate (%)	
	Mg	Mg + Na
0 (Native)	2.4 ± 0.6	1.8 ± 0.2
1.7	2.9 ± 0.3	2.5 ± 0.8
4.0	2.7 ± 0.5	1.6 ± 0.4
6.8	2.8 ± 0.2	2.6 ± 0.1

Table 7. Effect of different concentrations of oligomycin on the exchange rate in 4 experiments. Each tube contained 0.3 mM $MgCl_2$ and when used, 85 mM NaCl. Other ion concentrations and conditions were as described for fig. 4.

The definition of exchange rate is as for fig. 7.

reaction vessels were the same as those described in the Methods section -- i.e., Mg in one reaction vessel, and Mg + Na in the paired vessel containing another aliquot of the same microsomal pellet.

Figure 14 presents, in columnar form, the mean values ± SE from four determinations on the same batch of microsomes which had been incubated with the designated nucleotide, and ^{14}C-ADP.

The data on nucleotide preference of the Mg-dependent exchange rate are similar to those reported in electric eel microsomes (40) and rat brain microsomes (51). However, the Na-induced decrease in the nucleotide exchange rate in turtle bladder microsomes is in sharp contrast with that of a Na-induced increase in the nucleotide exchange rate for electric eel microsomes (40) and for rat brain microsomes treated with NaI (51).

In the presence of Mg alone, the order of nucleotide preference in terms of the exchange rate was: ATP, 17%; GTP, 3.8%; ITP, 3.5%; UTP, 2.7%; and CTP, 1.8%; which, in terms of percentage of the ATP rate, becomes ATP, 100; GTP, 22; ITP, 21; UTP, 16; and CTP, 11.

In the presence of Mg + Na, the above order was changed only slightly in that the preference for GTP and CTP was reversed. This effect on nucleotide preference was accounted for by the fact that sodium increased slightly the exchange rate between CTP and ADP. This small effect of sodium on the ADP:CTP exchange was the exceptional case, in that sodium had a retarding effect on the rate of exchange between ADP and all of the other nucleotides.

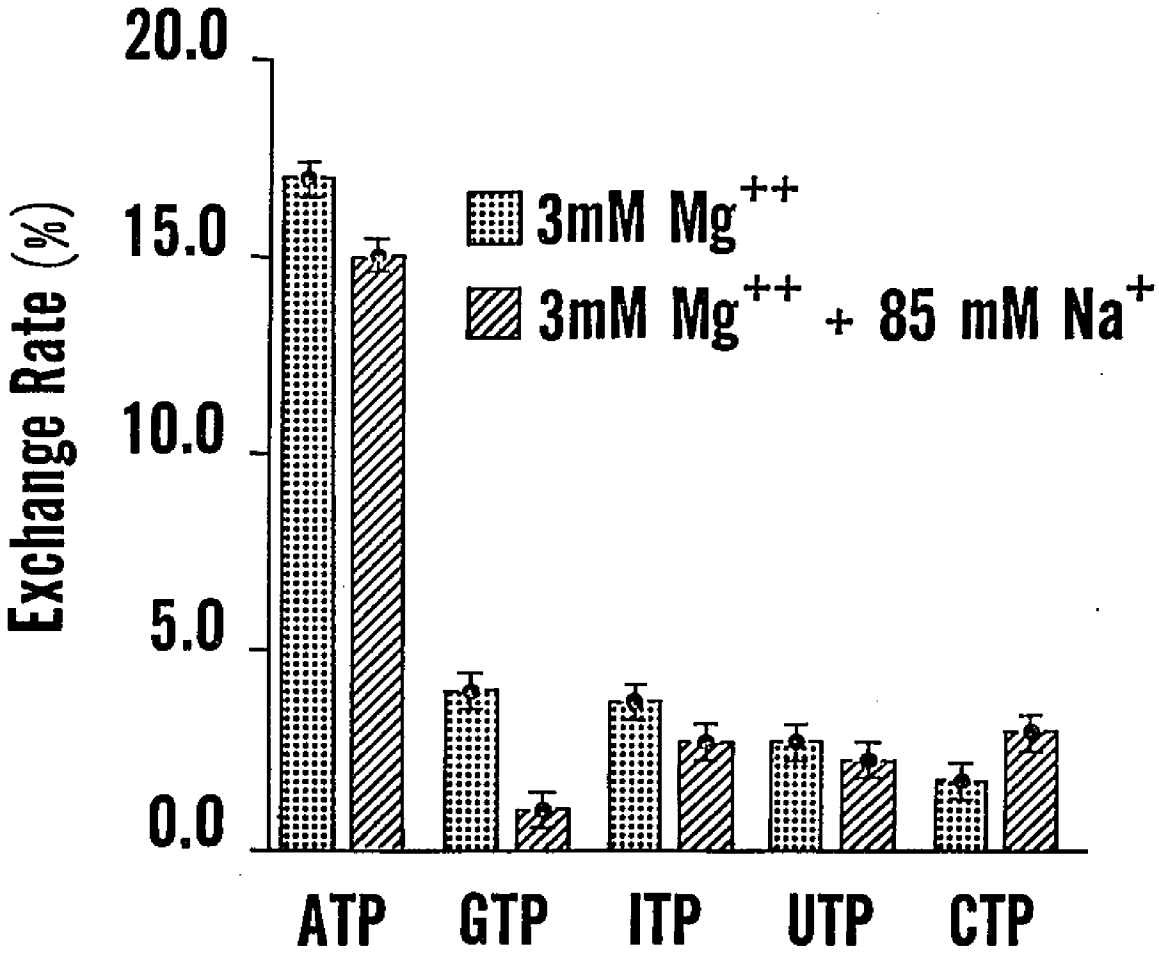

Fig. 14. Nucleotide specificity of the exchange rate. Equimolar concentrations of ITP, CTP, GTP, UTP (5 mM) instead of ATP. Ionic concentrations were 3 mM $MgCl_2$ and 85 mM NaCl when indicated. All ionic concentrations and conditions were as described for Fig. 10.

The geometric centers (mean values, n=4) and the vertical bars ($\pm$SE) are the same as described for fig. 9.

CHAPTER V

DISCUSSION

A. Microsomal Binding

The formation of intermediary protein complexes by the native and ouabain-treated forms of microsomal ATPase from turtle bladders can be related to transport phenomena in the intact tissue and to ATPase-activity in the isolated microsomal pellet. For example, ouabain, a known inhibitor of Na transport in the intact system and of (Na + K) -ATPase activity in the microsomes, has a three-fold effect on reactions involving the phosphorylated intermediate (a) It blocks the K-induced stripping of ^{32}p from the protein in the presence of Mg + Na; (b) It prolongs the time required for reaching the maximal level of ^{32}p-labelling in the presence of Mg + Na ("ouabain-shift"); (c) It retards the post-maximal rate of the K-independent stripping (of ^{32}p) from the protein. Although these effects (of ouabain on the phosphoprotein) resembled those usually reported for other tissues, a fourth effect (really the absence of an effect) is that the sodium-dependent increment of ^{32}p-labelling of the (ouabain-treated) protein was the same as that of the native protein during the first 50 seconds of incubation at 0°C. In short, ouabain apparently retards the rate but increases the amount of the (Mg + Na)-dependent-phosphorylation, and retards the dephosphorylation--even in the absence of K; and as expected from data on ATPase activity, it does not affect the Mg-dependent phosphorylation or dephosphorylation of the microsomal proteins.

The Na-induced decrement of the Mg-dependent ^{14}C-labelling of protein incubated with ^{14}C-ATP occurs simultaneously with an increment of the Mg-dependent ^{32}p-labelling of protein incubated with ^{32}p-ATP. These

data are taken as a direct demonstration of the formation and breakdown of a sodium-sensitive complex between ATP and microsomal protein, i.e. of the formation of a complex of the form, E-ATP in the initial step of the Na, K dependent ATPase reaction. A tentative scheme, consistent with the present data on Na-sensitive isotopic labelling of microsomal protein incubated with either ^{32}p-ATP or with ^{14}C-ATP, would only require the site of sodium action to be on reaction 4 (fig. 1). However to be consistent with the Na-induced decrement of the Mg-dependent exchange rate, it necessitates shifting the main site of sodium action to reaction 5 as has been discussed in detail when the exchange reaction data were introduced in the Results section.

The "sodium-sensitive" E-ATP complex has been demonstrated directly for the first time by the present experiments (see Tables 2, 3 and 5). This sodium-sensitive complex has been sought for but not found in guinea pig kidney microsomes (37) and in electric organ microsomes (50). Although Heinz and Hoffman did find Mg-dependent ^{14}C-ATP binding in erythrocyte ghosts, they made no mention of any effect of sodium (59).

The data from turtle bladder microsomes, like those from other tissues (6,14,37,40,50,58) are consistent with reactions 5 to 6 in fig. 2.

The present data, as well as those of others (6,14), on the effects of ouabain-treated microsomal proteins are in conformity with the aforementioned scheme of equations.

Thus, ouabain blocks the Na + K stimulated hydrolysis of ATP, and prevents the K-induced acceleration of the stripping of ^{32}p from microsomal proteins which had been incubated with ^{32}p-ATP. In the turtle bladder microsomes, ouabain failed to change the Na-induced increment

of ^{32}P-labelling of the protein in the first 50 seconds of incubation with ^{32}P-ATP at 0°C. This apparent negative result on the Na-induced increment was consistent with that found in crab microsomes incubated with ^{32}P-ATP at 37°C for more than 40 seconds, but was different from that in the same crab microsomes incubated for less than 40 seconds (14), and different from that found at 0°C in guinea pigs' kidneys (37) and electric organ of the eel (49). Despite the absence of a detectable change in the Na-induced ^{32}P-labelling after 50 seconds of incubation of ouabain-treated microsomes of turtle bladder, there was a prolongation (ouabain-shift) of the time required (from 60 to 160 seconds) to reach the maximal level of labelling.

In the case of binding of ^{14}C-ATP by ouabain-treated microsomes, only preliminary experiments have been conducted thus far. Results therefrom, tentative at the best, do suggest that the Na-induced decrement of Mg-dependent ^{14}C-labelling is still present.

B. Exchange reaction

What can be said on the basis of the present data on the catalyzed exchange rate is that ADP can be phosphorylated by a high energy phosphoprotein and that this reaction, as well as those of enzyme substrate complexing and overall hydrolysis, is highly dependent upon the concentration of Mg, Na and K in the ionic environment. Whereas the microsomal enzyme mixture may have ionic requirements identical to those of the intact transport system, it lacks the spatial orientation which provides directionality for the active transport process.

The reaction model (fig. 2) like any enzyme-substrate reaction model, requires that the enzyme be regenerated after formation of the final reaction product P_i, which means that the sodium form, E', is

converted to the original form, E. This possibility can be expressed by the reversible reaction,

$$E' \rightleftharpoons E,$$

where the Mg-dependent enzyme is assumed to consist of two isozymes which might exist in a paired association throughout the entire sequence of binding, phosphorylation, and hydrolysis. This notion of paired isozymes, analogous to the R and T forms of the protein in the Monod-Wyman-Changeux model(36),may be used to explain the variations in Mg dependent and Na, K-dependent activities in microsomal preparations from various tissues. For example, the high ratio of Na + K/Mg activity in the eel microsomes (40) might be ascribed to a high equilibrium ratio of E' to E. The concept may be used to explain the separation of a Mg-dependent, Na-independent exchange activity in rat brain microsomes (64) where the Na + K dependent moiety can be correlated with a high equilibrium ratio of E' to E, and the Na-independent moiety, with a low equilibrium ratio.

The reaction model scheme (fig. 2) is by no means the complete mechanistic picture of the Na, K dependent ATPase. Nevertheless, it is suggestive of the existence of ATPase coupling to the carrier-operation and ion transport as well as to the energy metabolism of the cell.

C. Problems remaining

Problems remaining to be solved include: (a) the further separation of Mg-dependent from Na + K dependent activity; (b) the nature of the intermediary complexes formed; and (c) the exact role of membrane ATPase in active Na transport.

(a) The high proportion of Mg-dependent activity in hydrolysis and binding as well as in transphosphorylation may well be ascribed to the presence of a Mg-dependent, sodium-independent protein activity which

may or may not be related to "transport ATPase" in the turtle bladder. Further purification of the microsomal protein mixture, such as has been attempted by others (64, 65) is still required for a more exact biochemical interpretation.

(b) Available data on Na-induced decrements in the exchange rate and in the amount of ^{14}C-ATP complex formed are sufficient to invoke the existence of an E-ATP complex, but are not sufficient to describe the physical chemical nature of such an association, moreover present data are not sufficient to determine whether Na acts on the Mg form of the enzyme (E) or only on the Na form of the enzyme (E'). Data on phosphoprotein formation and breakdown involve unspecified interactions among Na, K, ouabain, and phosphoproteins. The analysis of such interactions is currently under scrutiny here and elsewhere.

(c) The (Na + K)-sensitivity of a membrane located ATPase activity appears analogous to the cation-selectivity of the binding between Na and a carrier protein during the process of active transport in the physiologically intact system.

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